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SOIL & FERTILIZER

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Purification and Some Properties of the Lipase and Phospholipase of *Sclerotinia* Fungus

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Received September 5, 1959

The lipase and phospholipase of *Sclerotinia Libertiana* extracted from the cells with a dilute ammonium hydroxide solution could be fractionated by precipitation and adsorption-elution methods, and a partial purification of the lipase removed the activity towards Tween 20.

From the examination of hemolysis and of phosphorus release from lecithin, the phospholipase was shown to involve types A,B and C. Phospholipase A was purified and obtained in crystalline form. The heat-stability of phospholipase A and C, and substrate specificity of the two lipases are also presented in this paper.

INTRODUCTION

Recent investigations have established that the product of hydrolysis of triglyceride by pancreatic lipase is the 1,2-di or 2-monoglyceride[1], and Tween is not assumed to be a substrate for the purified lipase[2]. On the other hand, in a previous paper[3] the authors demonstrated that a mixture of a preparation of *Sclerotinia* lipase and pancreatic lipase readily hydrolyses more than 60% of olive oil. The fungal preparation was active towards substrates other than glycerides such as Tween. The activity towards Tween seemed to have an important role on the hydrolysis of triglycerides by a combination of the two enzymes.

Though the enzymic degradation of lecithin had been recognized to proceed in two steps by phospholipase A and B, preparations of the phospholipase B from *Pen. notatum*[4] or rat intestinal mucosa[5] have been found to split the two-fatty acids from lecithin, if certain acidic lipids were added. The phospholipase system of the *Sclerotinia* fungus was shown previously to hydrolyse lecithin directly, but the type of enzymes involved have not yet been demonstrated. The type of phospholipase has a close connection with hemolysis.

Thus, purification of the lipase and phospholipase of *Sclerotinia* fungus was undertaken, and principles of the action of the phospholipase were investigated in an examination of hemolysis.

EXPERIMENTAL AND RESULTS

Enzyme source; The dry powder of a bran culture, or the freeze-dried mycelium of a submerged culture, of *Sclerotinia Libertiana* was used.

Extraction: The bran culture was extracted with ten volumes of N/20 ammonium hydroxide, and the mycelium was extracted twice with N/40 ammonium hydroxide.

Enzyme activity; The lipase and phospholipase activities were measured by the method reported in the previous paper[3] and expressed as ml of N/20 sodium hydroxide solution required with either 1 ml of olive oil or Tween, or 0.5 g of soy-lecithin as substrate. Release of acid-soluble phosphate from lecithin by the phospholipase was determined according to the method of MacFarlane and Knight[6].

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* Osaka Bacteriological Research Institute Ltd.

[1] B. Borgstrom, *Biochim. Biophys. Acta*, **13**, 491 (1954); F.H. Mattson and L.W. Beck, *J. Biol. Chem.*, **214**, 115 (1955); P. Desnuelle and P. Savary, *C.R. Ac. Sci.*, **240**, 2571 (1955); N.H. Taffric, R.A. Bailey and M. Kates, *Arch. Biochem. Biophys.*, **78**, 319 (1958).

[2] L. Sarda, G. Marchis-Mourex and P. Desnuelle, *Biochim. Biophys. Acta*, **30**, 224 (1958).

[3] This Bulletin, **23**, 150 (1959).

[4] R.M.C. Dawson, *Biochem. J.*, **70**, 559 (1958).

[5] B. Epstein and B. Shapiro, *Biochem. J.*, **71**, 615 (1959).

[6] M.G. MacFarlane and B.C.J.G. Knight, *Biochem. J.*, **35**, 884 (1941).

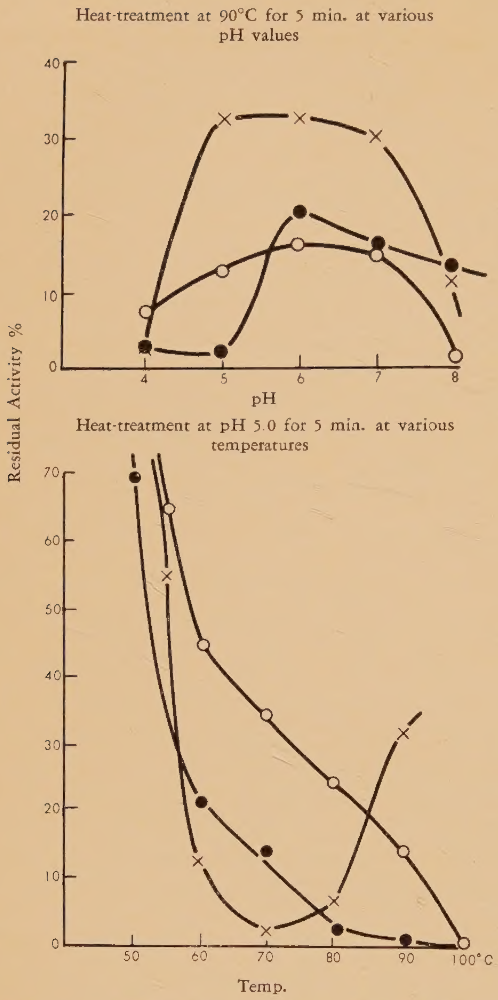


FIG. 1. Heat Stability of Lipase and Phospholipase

-○——○- on olive oil
-×——×- on lecithin
-●——●- on Tween 20

Hemolysis; Hemolysis was tested on bovine erythrocytes by Slotta's method[7].

Partial Purification of Lipase

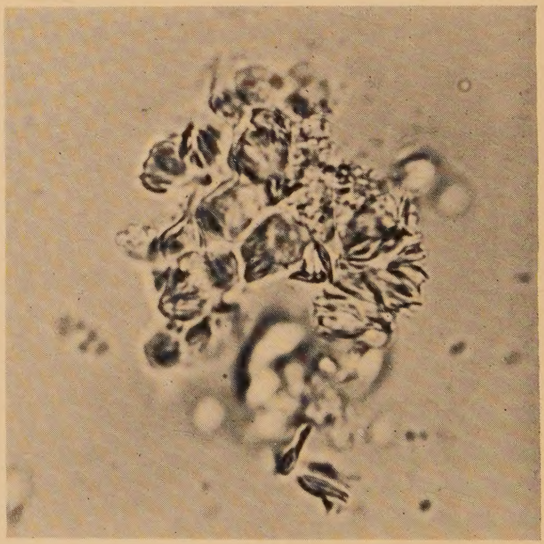
As shown in Table I, the lipase extracted from the mycelium of a submerged culture was found to be precipitated by dilute acetic acid and could be redissolved in dilute ammonium hydroxide. After repeating the precipitation and dissolution, colored impurities in the lipase solution were removed by adding a small amount of rivanol and passing the solution through celite. Thereby, activity of the lipase to olive oil was found to be increased about 30 fold. Whereas, the activity to Tween 20 had almost disappeared by this stage, and the phospholipase activity had been greatly reduced.

Heat Stability

Heat-stability of the lipase and phospholipase was investigated with an original extract from the mycelium. As seen in Fig. 1, the activity of lipase was found to have a different heat-stability for olive oil and for Tween 20, and after heating at 90°C for five minutes, phospholipase was more stable than lipase between pH 5 and 7. Also, it was found that the heat-stability of phospholipase for five minutes is higher at 90° than 60～80°.

Purification of Phospholipase

Phospholipase was purified by the procedure presented in Table II. The phospholipase in the extract of a bran culture was found to be specifically precipitated by lead acetate and extracted with phosphate buffer (pH 5.2). Subsequently, this phospholipase extract was purified to some extent by absorption on calcium phosphate and elution by phosphate buffer containing ammonium sulphate. After dialysis the fraction was concentrated by freeze-drying, and, after further dialysis against ammonium sulphate, was again concentrated *in vacuo* to a small volume. The concentrated solution, saturated with ammonium sulphate, was filtered through paper and stored in



×600

Photo. 1

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[7] K.H. Slotta and G. Szyzka, *Ber.*, **71**, 258 (1938).

a refrigerator for 2 or 3 days. The crystalline precipitate, seen in Photo. 1, then appeared. This precipitate was dissolved and it had 35 times more specific activity than the original. The activity was measurable in ether[8] and responsible for phosholipase A. However, there was still some lipase activity towards olive oil.

Hemolytic Effect and Release of Acid-soluble Phosphate

The hemolytic effect was examined in an extract from a bran culture. As shown in Table III, degradation of lecithin by an appropriate amount of the extract was found to cause hemolysis at a high degree of dilution. But, when the lecithin was decomposed by a somewhat high concentration of the extract, hemolysis no longer occurred. The result may show that the lysolecithin

TABLE I. PARTIAL PURIFICATION OF LIPASE

Procedure	Volume in ml	Activity on olive oil per ml,	per mg N content	Activity on Tween 20, per ml	Phospholipase per ml
Extract of the mycelium grown in submerged culture (1 ml=50 mg of mycelium)	500	11.3	0.8	6.0	9.0
Precipitated at pH 5~6 with dil. acetic acid, and dissolved in dil. ammonium hydroxide	500	10.0	6.4	3.0	4.0
Repeated twice	100	28.0	22.0	—	—
Centrifuged after adding 0.05% rivanol at pH 7.5	100	29.0	25.0	—	—
Filtered through celite	100	31.0	27.0	0	1.0

TABLE II. PURIFICATION OF PHOSPHOLIPASE

Procedure	Volume in ml	Phospholipase activity per ml as below	per mg N content	(Lipase activity on olive oil) per ml
Extract of bran culture (1 ml=100 mg of dry culture)	1810	8.5/2 ml	2.8	8.0
Precipitated with 2% lead acetate. Washed with water. Repeatedly extracted with M/15 phosphate buffer (pH 5.2)	1000	6.5/2 ml	—	2.0
Calcium chloride soln. and celite added. Adsorbed on calcium phosphate and eluted with M/10 phosphate buffer (pH 7.0) containing 20% ammonium sulphate	1000	4.8/2 ml	30.0	1.3
Dialysed against water for 1 day. Concentrated by freeze-drying	200	13/1 ml	—	5.0
Dialysed against 20% ammonium sulphate for 2 days				
Concentrated *in vacuo*. Filtered and stored in ice box	29			
Crystalline precipitate dissolved to	29	12/02 ml*	100	4.0
Filtrate	29	6/0.2 ml	—	1.5

* The activity was also measurable in ether, showing a value of 4.25 and 5.5 per 0.2 ml respectively by methods (I) and (II) of Hanahan.

---

[8] D.J. Hanahan, *J. Biol. Chem.*, **195**, 199 (1952); D.J. Hanahan, M. Rodbell and L.D. Turner, *J. Biol. Chem.*, **206**, 431 (1954).

TABLE III. HEMOLYTIC EFFECT

i. Hemolysis by enzymic degradation product of lecithin.

Soy-lecithin was decomposed by the *Sclerotinia* enzyme as in the method previously reported. The reaction mixture containing 50 mg/ml lecithin initially was diluted as follows, and 1 ml of the diluent was mixed with 0.2 ml of M/20 phosphate buffer (pH 7.4) and 1 ml of 4% bovine erythrocytes suspension and was incubated at 37°C for 2 hrs.

Enzyme preparation used for lecithin decomposition		Dilution of the reaction mixture	Hemolytic effect
Extract of bran culture	2 ml	1/1000	+
〃	4 ml	1/1000	−
Extract heated at 90°C for 3 min.	4 ml	1/100	+
Extract heated at 100°C for 60 min.	4 ml	1/100	−

ii. Hemolysis by enzyme solution alone

Enzyme solution mixed with phosphate buffer and erythrocytes suspension as in the above.

Enzyme preparation added		Hemolytic effect
Extract of bran culture	2 ml	+
〃	1 ml	−
Extract heated at 100°C for 60 min.	2 ml	−
Extract heated at 90°C for 3 min.	2 ml	+
〃	1 ml	+
〃	0.5 ml	±

TABLE IV. RELEASE OF ACID-SOLUBLE PHOSPHATE FROM LECITHIN

Substrate	Enzyme solution added		P. released (mg/ml)
Soy-lecithin	Extract of bran culture	2 ml	0.108
〃	Extract heated at 90°C for 5 min.	2 ml	0.075 (Residual activity 70%)
Egg-yolk	Extract of bran culture	2 ml	0.324
〃	Extract heated at 90°C for 5 min.	2 ml	0.129 (Residual activity 40%)

Reaction mixture consisting of 1 ml 2.5% soylecithin soln. or egg-yolk, 2 ml enzyme soln., 0.2 ml 0.3 M $CaCl_2$ soln., 1 ml borate buffer (pH 7.1) and 0.8 ml dist. water was incubated at 37°C for 15 min. and after the addition of 1 ml trichloroacetic acid (20%), acid-soluble P was measured. The control value was subtracted.

produced by phospholipase A was further decomposed by phospholipase B. Whereas, the effect was still observed even with a large amont of the extract, which had been heated at 90°C for three minutes. In this case, phospholipase A alone must be participating.

Also, hemolysis occurred without addition of lecithin, when the extract was directly mixed with erythrocytes in the ratios shown in the Table III–ii. The effect was considered to be due either to the action of phospholipase A or C.

As shown in Table IV, acid-soluble phosphate released from lecithin by the extract was measured. Thus the action of phospholipase C was confirmed. Also, the heat-stability of the phospholipase C was higher than A.

DISCUSSION

The high lipase activity of a *Sclerotinia* preparation towards Tween 20 was shown not to be due to the action of the normal lipase, but to another specific lipase. Studies are in progress on the mechanism of the increased effect of combination of these two lipases on hydrolysis of triglycerides, and some results will be reported in the following paper.

Sclerotinia phospholipase was proved to involve types A, B and C. Lecithin was degraded by the enzyme in McIlvane's buffer (pH 6) in experiments on hemolysis. The action of phospholipase C was retarded to some extent by citrate and phosphate[6]. Accordingly, it is reasonable to assume that the hemolysis was due to lysolecithin.

However, hemolysis by the direct action of the enzyme seems to be complicated by the action of phospholipase A and C, both of which are participating to produce the hemolysis. The direct hemolytic effect observed after heating the enzyme could be ascribed to the action of phospholipase C.

The phospholipase A in a crude extract is rather more stable for five minutes at 90° than at 60°. Similar result has been reported by Kushner[9] with a bacterial lecithinase which corresponds to phospholipase C.

Crystalline crotoxine, formerly believed to be a single protein, has recently been resolved into two components[10]. The existence of several protein fractions with phospholipase A activity has also been demonstrated in snake venoms[11~14]. On

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9) D.J. Kushner, *J. Bacteriol.*, **73**, 297 (1957).

10) H. Fraenkel-Conrat and B. Singer, *Arch. Biochem. Biophys.*, **60**, 64 (1956).

11) W. Grassman and K. Hannig, *Z. Physiol. Chem.*, **296**, 30 (1954).

12) C.C. Yang, S. Iwanaga and S. Kawachi, *J. Formosan Med. Assoc.*, **57**, 525 (1958).

13) A. Ohsaka, *J. Biochem.*, **45**, 259 (1958).

14) S. Iwanaga and S. Kawachi, *J. Pharm. Soc. Japan*, **79**, 582 (1959).

properties of the fungal phospholipas A obtained in crystalline form, further investigations are necessary.

The authors wish to express their sincere thanks to Prof. I. Yamasaki for reading this manuscript.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 333~337, 1960]

Chemical Studies on Actin

Part I. On the Changes of Bindings of Actin to Alkaline Earth Metals during Aging of Meats

By Masao FUJIMAKI, Nobuhiko ARAKAWA and Hiroko YONETA

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Received September 15, 1959

The content of alkaline earth metals in actin during aging of meats was determined. The content in actin was found to be maximum immediately after slaughter and minimum at two days of aging. The rate of calcium to alkaline earth metals except calcium was high immediately after slaughter, but became low during aging.

As the content of alkaline earth metals in actin extracted from the acetone dried actomyosin has been found to be less than that from the same muscle, the result obtained appears to show that in meats actin exists in both, free and bound form, the latter being one constituent of actomyosin, and at the process of aging in which minimum content of alkaline earth metals in actin was observed, actin was more in the form of actomyosin than at other process.

As reported by Guba and Szent-Györgyi(1), actin has been prepared from the water extractive solution of acetone dried powder after removal of myosin fraction from the muscle.

However recently Bárány et al.(2) have reported that actin can be extracted with univalent salt solution containing adenosine triphosphate (ATP) from the acetone dried muscle powder without previous extraction of myosin.

To remove alkaline earth metals from the muscle, it was washed repeatedly with 0.4% sodium bicarbonate solution.

As shown in the report, it has been considered that actin is bound to myosin through alkaline earth metals in the acetone dried muscle powder and by splitting this binding with salt solutions containing univalent ions, actin changed into free form and became possible to be extracted.

Therefore, in order to study the existing condition of actin in meats, actin was prepared during aging of meat by the method of Bárány et al. and the contents of free and bound alkaline earth metals in the acetone dried muscle powder and actin were determined. The results obtained are as follows.

---

1) F. Guba, A. Szent-Györgyi, *Nature*, **158**, 343 (1946).
A. Szent-Györgyi, Chemistry of Muscular Contraction, 2nd ed., Academic Press, (1951) p. 148.

2) M. Bárány et al., *Acta Physiologica* (Academiae Scientiarum Hungaricae), **11**, 145 (1957).

EXPERIMENTAL

Preparation of Actin

Preparation of actin was carried out as follows by the method of Bárány et al. To the required weight of the muscle of rabbit (Longissimus dorsi) or cow (neck) were added 20 volumes of 0.4% sodium bicarbonate solution and the mixture was stirred for 20 minutes at room temperature and centrifuged, and the supernatant was discarded. Again the same volumes of 0.4% sodium bicarbonate solution were added to the residue and the mixture was stirred and centrifuged repeatedly.

Thereafter, 15 volumes of distilled water were added to the residue and the same procedure was carried out as previously. Ten volumes of acetone were added to the residue and the mixture was filtered by suction after stirring.

Similarly, 3 volumes of acetone were added to the residue and the same procedure was carried out four times successively.

This acetone treated residue was allowed to stand over night at room temperature and dried. Twenty-five volumes of 0.3 M potassium iodide solution containing 5×10^{-4} M ATP were added to the definite quantity of the acetone dried muscle powder. After the extraction for 30 minutes, the mixture was centrifuged, and to the supernatant were added 9 volumes of distilled water and one-hundredth volume of 1 M acetate buffer (pH 4.7).

The mixture was allowed to stand for 10 to 20 minutes in order to precipitate the actin, and was centrifuged. To the precipitate (crude actin) was added the same volume of 0.3 M potassium iodide solution as above mentioned and actin was dissolved. Thereafter, the procedure was carried out twice repeatedly. The supernatant was confirmed not to contain alkaline earth metal ion.

Finally, the precipitate was dissolved by the addition of 0.3 M potassium iodide solution and used as refined actin solution.

The quantitative determination of alkaline earth metals was carried out by the method of Nancy Arnold et al.[3]. To the sample ammonium buffer solution (the mixture of 1 volume of 1 M ammonium chloride with 5 volumes of 1 M ammonium hydroxide solution) and a drop of Eriochrome Black T solution (2 g of Dotite B.T.* is dissolved in 50 ml of methanol containing 2 g of hydroxylamine hydrochloride) were added. Then, the solution was titrated with 1×10^{-3} M EDTA solution (sodium salt of ethylene diamine tetraacetic acid).

The quantitative determination of calcium was carried out by the method of Tenness[4].

To the sample 2 N sodium hydroxide solution was added to make the pH of the solution above 10, and it was titrated with 1×10^{-3} M EDTA solution using about 0.2 g of Dotite N.N.** as the indicator.

RESULTS AND DISCUSSION

In order to investigate the error for the determination of calcium and alkaline earth metals, 1×10^{-3} M $CaCO_3$ and 1×10^{-3} M $MgCl_2$ solution were titrated with 1×10^{-3} M EDTA solution using Dotite B.T. and Dotite N.N. as indicators respectively.

The results obtained are shown in Table I. When Dotite B.T. was used as the indicator, the total titration values almost completely corresponded the sum of each titration value, irrespective of the concentration of calcium and magnesium ions. In the case of Dotite N.N. as the indicator, with which only calcium ion was titrated, the titration values seemed to increase in the presence of magnesium ion than in the absence of that ion. However, when the concentration of calcium is higher than that of magnesium, the error in the titration appears to decrease.

TABLE I. TITRATION ERROR IN THE DETERMINATION OF CALCIUM AND MAGNESIUM

$CaCO_3$ solution*	$MgCl_2$ solution**	Titration value (ml) of 1×10^{-3}M EDTA solution	
		Dotite B.T.	Dotite N.N.
ml	ml		
1	0	0.99	1.00
0	1	0.84	0.00
1	1	1.84	1.16
1	2	2.70	1.16
1	3	3.55	1.16
2	1	2.77	2.30
3	1	3.75	3.11

* 1×10^{-3}M ** ca. 1×10^{-3}M

It has been reported that in order to eliminate the interfering effects of phosphate and sulfate on this titration, the preliminary treatment of the sample through anion exchange resin is essential.

---

[3] Nancy Arnold et al., *Food Technol.*, **10**, 245 (1956).

[4] R. Tenness, *Anal. Chem.*, **25**, 967 (1953).

* 1-(1-Hydroxy-2-naphthylazo)-5-nitro-2-naphthol-4-sulfonic Acid Sodium Salt

** 2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic Acid

But when the muscle extract solution digested with nitric acid was passed through the column of Amberlite IR-4B on hydrochloric acid form, there was found little difference in the titration value between the sample and untreated one. Hence the ion-exchange treatment was not adopted. As the quantity of alkaline earth metals extracted from muscle during the preparation of acetone dried muscle, is shown in Table II, no difference was shown in the quantity of free alkaline earth metals (considered mainly as calcium and magnesium) extracted from muscle during aging of meats. But the quantity of total alkaline earth metals released from the nitric acid digestion increased obviously during aging of meats.

TABLE II. THE QUANTITY OF ALKALINE EARTH METALS EXTRACTED FROM MUSCLE DURING THE PREPARATION OF ACETONE DRIED MUSCLE (mM/100g of muscle)

Aging period (day)	Alkaline earth metals Free	Alkaline earth metals Total
0	1.01	1.52
2	1.21	1.65
4	1.28	1.90
6	1.11	1.90
8	1.26	1.94

In this table, the quantity of free alkaline earth metals seemed to show the sum of alkaline earth metals which existed in free form and changed free by substitution with sodium bicarbonate. The difference in the quantity of total and free alkaline earth metals was considered to show the quantity of alkaline earth metals bound to protein.

TABLE III. THE QUANTITY OF CALCIUM EXTRACTED FROM MUSCLE DURING THE PREPARATION OF ACETONE DRIED MUSCLE (mM/100g of muscle)

Aging period (day)	Free calcium	Total calcium
0	0.20	0.27
2	0.21	0.43
4	0.26	0.27
6	0.25	0.27
8	0.21	0.27

The quantity of calcium extracted from muscle during the preparation of acetone dried muscle powder is shown in Table III. The majority of alkaline earth metals in muscle are considered to be calcium and magnesium, and the quantity of magnesium extracted from muscle during the preparation of acetone dried muscle powder is much more than that of calcium.

The quantities of free and total calcium showed little changes during aging of meats except at two days of aging. As there are little differences in the quantity of total and free calcium, calcium seemed not to be bound to the protein (perhaps globular protein, myogen) extracted from muscle during the preparation of the acetone dried muscle powder.

Bárány et al. have reported that in the acetone dried muscle powder, actin is bound to myosin through alkaline earth metals and actin is extracted by splitting this binding, and that for splitting this binding, 0.3 M potassium iodide solution containing 5×10^{-4} M ATP and 2×10^{-2}M phosphate buffer (pH 7.0) is the most suitable solution.

When actin is extracted from the acetone dried muscle with the solution as previously mentioned, the quantity of alkaline earth metals released is shown in Table IV, the quantity being the minimum at two days of aging. Considering the results in Table V, not all free

TABLE IV. THE QUANTITY OF ALKALINE EARTH METALS RELEASED, WHEN ACTIN IS EXTRACTED FROM THE ACETONE DRIED MUSCLE (μM/acetone dried muscle g)

Aging period (day)	Alkaline earth metals	Calcium	The content of acetone dried muscle from 100g of muscle
0	10.61	4.43	15.56
1	10.11	4.06	13.52
2	9.97	4.13	12.31
4	10.69	3.81	12.42
8	10.78	4.03	9.83

TABLE V. THE QUANTITY OF ALKALINE EARTH METALS RELEASED, WHEN ACTIN IS EXTRACTED FROM BOTH THE ACETONE DRIED COW'S MUSCLE AND THE ACETONE DRIED ACTOMYOSIN (L) OF THE SAME MUSCLE
(μM/acetone dried powder g)

	Alkaline earth metals	Calcium
Acetone dried muscle	10.40	4.06
Acetone dried actomyosin (L)	5.67	2.48

alkaline earth metals shown in Table IV are supposed to take part in combination of actin and myosin.

In Table V, is shown the quantity of alkaline earth metals released, when actin is extracted from both the acetone dried powder prepared from the muscle of cow and the acetone dried actomyosin (L)[5] from the same muscle.

According to Bárány et al., actin can be extracted from actomyosin as well as from muscle. As shown in Table V, the quantity of alkaline earth metals released when actin is extracted from the acetone dried actomyosin is much less than that from the acetone dried muscle powder.

So, it has been supposed that as the proportion of actomyosin in the acetone dried muscle powder increased, the quantity of alkaline earth metals released in the extraction of actin decreased. Also, the decrease of released alkaline earth metals at two days of aging as shown in Table IV will indicate the maximum formation of actomyosin, which has been demonstrated by the authors[6] in the previous study on the aging of meats. As shown in Table IV and V, the quantity of magnesium is more than that of calcium among released alkailne earth metals. It is noticeable that there are little differences between the quantity of released alkaline earth metals from the acetone dried muscle powder of rabbit and cow.

TABLE VI. THE QUANTITY OF ALKALINE EARTH METALS RELEASED, WHEN ACTIN IS EXTRACTED FROM THE ACETONE DRIED ACTOMYOSIN (L) OF COW'S MUSCLE (μM/g)

Kind of extractive solution	Alkaline earth metals
Redistilled water	1.91
Potassium iodide solution	7.64
Potassium iodide solution containing ATP	7.46

TABLE VII. THE CONTENT OF ALKALINE EARTH METALS IN ACTIN* (M/57,000 g)

Aging period** (day)	Alkaline earth metals	Calcium
0	4.62	2.83
1	3.65	1.17
2	3.01	0.93
4	3.66	1.23
8	3.96	1.33

* Prepared from rabbit's muscle
** The temperature during aging was held at about 1°.

When actin is extracted from the acetone dried muscle powder, the relation between the kind of the extractive solution and the quantity of released alkaline earth metals is shown in Table VI. The quantity of alkaline earth metals released by the extraction with the distilled water is much less than that with potassium iodide solution.

In this case, the addition of ATP has had little influence on the released quantity of alkaline earth metals. This fact will support the theory of Bárány et al. that actin is extracted by salt solutions containing univalent ions, but it may still be necessary to examine this in detail in future.

The content of alkaline earth metals in actin is shown in Table VII. Alkaline earth metals in actin do not exist in free form, but in bound form, being released by digestion with the nitric acid.

As shown in Table VII, the content of calcium in actin, which is prepared from muscle immediately after slaughter, is 2.83 M per 1 M of actin (57,000 g[2]). This quantity nearly agrees with the one presented by Guba which is 1 M per 18,000 g of actin[7].

The content of alkaline earth metals in actin during aging of meats is shown to be maximum immediatedly after slaughter and minimum at two days of aging. The rate of calcium to

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[5] M. Fujimaki, *J. Agr. Chem. Soc. Japan*, **32**, 689 (1958).
[6] M. Fujimaki and Y. Nakajima, *ibid.*, **32**, 695 (1958).
[7] A. Szent-Györgyi, Chemistry of Muscular Contraction, 2nd ed., Academic Press, (1951) p. 69.

TABLE VIII. THE CONTENT OF ALKALINE EARTH METALS IN ACTIN PREPARED FROM ACETONE DRIED COW'S MUSCLE AND ACTIN FROM ACETONE DRIED ACTOMYOSIN (L)
(M/57,000 g)

	Alkaline earth metals	Calcium
Actin prepared from muscle	6.47	3.09
Actin prepared from actomyosin (L)	2.03	1.47

alkaline earth metals except calcium is high immediately after slaughter, but becomes low during aging.

As shown in Table VIII, it has been found that the content of alkaline earth metals in actin extracted from the acetone dried actomyosin L is less than that of the same muscle, so the minimum content of alkaline earth metals in actin seems to show that at this stage of aging actin has been more in the form of actomyosin than at other process.

The content of alkaline earth metals in actin which is prepared from the muscle of cow by the method of Bárány et al. and that which is prepared from the actomyosin L of the same muscle is shown in Table VIII. In actin prepared from actomyosin L, the content of alkaline earth metals is very small, but the rate of calcium occupied in alkaline earth metals seemed to be large.

Comparing with the results in Table VII, the contents of alkaline earth metals in actin prepared from the muscle of rabbit are less than that prepared from the muscle of cow. Therefore, it can be considered that the chemical constituents of actin may be different from the kinds of animals, and it will be necessary to examine in detail this question in future. The result that the content of alkaline earth metals in actin prepared from actomyosin is less than that in actin prepared from the same muscle, appears to show that in muscle actin exists in both, free and bound form, the latter being one constituent of actomyosin and the change of these existing form in muscle is confirmed from the determination of the content of alkaline earth metals, assuming that the content in free actin (actin prepared by the method of Guba and Szent-Györgyi) is constant during aging of meat.

Acknowledgement. The authors wish to express their thanks to Prof. Y. Sakurai for his interest and encouragement in their work. This experiment has been supported in part by grant from the Ministry of Education.

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Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part II. Relation between Adsorption of Acid Antiseptics on Yeast Cell and their Toxic Effect

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The acid antiseptics (salicylic, benzoic, dehydroacetic and sorbic acid) transferred to the yeast cell exist in two states, i.e., the adsorbed one on the solid phase of the cell and the dissolved one in the inner cell fluid.

The adsorbed quantity of the antiseptics increases by the addition of inorganic salts in the medium. The toxic effect on the yeast also increases with the increase of the salts concentration, being independent of the biological effect of the salts themselves. On the other hand, the dissolved quantity changes with the content of inner cell water which can be controlled by the physical action of the osmotic pressure in the medium. However, the toxic effect is independent of the change of the dissolved quantity.

As the conclusion, it is considered that the adsorbed quantity is the limiting factor of the toxic effect.

In the previous report[1], it has been suggested that the acid antiseptics transferred to yeast cells exist in two states: one is the dissolved antiseptic in the inner cell fluid and it is in the ionization equilibrium as proposed by Conway and Downy[2], and the other is the adsorbed one on the solid phase of the cell.

The existence of the above mentioned two states was confirmed by controlling the water content of the cell by the physical action of the osmotic pressure.

Each role of the adsorbed antiseptic and dissolved one in the toxic effect was examined and it was considered that the adsorbed quantity is the limiting factor of the toxic effect of the antiseptics. These results are presented in this report.

EXPERIMENTALS

The experiments were carried out with washed bakers' yeast, and the water content in the cell was 65.4 percent.

The antiseptics tested were salicylic, benzoic, dehydroacetic and sorbic acid.

Determination of transferred quantity. The transferred quantity of antiseptics was determined in the buffer solution at 30°C. The method of the determination was described in the previous report[1] and was not modified in any detail.

Evaluation of water abstracted from yeast cell by osmotic pressure. The haematocrit method of Conway and Downy[3] was used, and the method is concerned with the measurement of the centrifuged volume of the cells in a thick-walled glass capillary.

Growth curve of yeast. Koji extract (Ballg. 7°) containing test material was inoculated with the washed yeast in 0.01 percent, and then incubated at 30°C. After a definite interval of time, a part of the culture was taken out and centrifuged. The centrifuged yeast was repeatedly washed and suspended in distilled water. Then, the optical extinction of this suspension was measured at the wave-length of 350 mμ, and the

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1) S. Oka, This Bulletin, **24**, 59 (1960).

2) E.J. Conway and M. Downy, *Biochem. J.*, **47**, 355 (1950).

3) E.J. Conway and M. Downy, *Biochem. J.*, **47**, 347 (1950).

extinction value was plotted against the time.

Determination of toxic effect of antiseptics. The minimum concentration of antiseptics required to inhibit the growth of bakers' yeast was determined.

The basal medium was koji extract (Ballg. 14°), the pH of which was adjusted to a desired value with hydrochloric acid or sodium hydroxide. In a test tube, 5 ml of the basal medium was mixed with 5 ml of the antiseptic solution, the pH value of which was adjusted to the same value as the basal medium. When the medium of high osmotic pressure was necessary, a difinite amount of unhydrous glycerin, glucose or solid inorganic salts was added to the mixture. In this case, the concentration of the solutes was represented by mole per kg of water in the medium.

Mixtures containing the antiseptic in various concentrations were inoculated with one drop of the yeast suspension containing about 0.1 percent of the washed cell, and were incubated at 30°C. Then, the growth was observed with naked eyes by the turbidity of the cultures after two days of the incubation, and at the same time, the final pH of the medium in which the yeast did not grow was measured by the glass-electrode pH meter.

RESULTS AND DISCUSSION

Transferred quantity under inhibiting condition. The minimum concentration required to inhibit the yeast growth greatly changes with the pH value in the culture medium as shown in Table I, and also the values of k and n in equation (1)1) change with the pH as shown in the same table. Equation (1) expresses the relation

$$a=k\,(C_m)^n \qquad (1)$$

between the quantity of the antiseptics transferred to the cell, a (m-mol./kg-cell), and the equilibrium concentration in the medium, C_m (m-mol./kg-water).

From equation (1), the transferred quantity under the inhibiting condition can be evaluated by substituting the observed inhibiting concentration in C_m, because the values of k and n under the same condition have been given in Table I.

It is noteworthy that the evaluated transferred quantities in the last column of Table I and the concentrations of the undissociated antiseptics

TABLE I. INFLUENCE OF pH ON TOXIC EFFECT AND TRANSFERRED QUANTITY OF ACID ANTISEPTICS

Antiseptic	pH in medium	Minimum conc. for inhibition of yeast growth (m-mol./kg-water)	Conc. of undissociated antiseptic (m-mol./kg-water)	k	(n)	Transferred quantity of antiseptic (m-mol./kg-cell)
Dehydroacetic acid pK : 5.1	3.06	0.3	0.30	6.9	(0.78)	2.7
	4.05	0.5	0.46	8.3	(0.89)	4.5
	5.00	2	1.1	3.6	(0.86)	6.6
	6.03	7	0.73	1.6	(0.94)	10
Sorbic acid pK : 4.7	3.02	2	2.0	13	(0.68)	21
	4.00	4	3.3	8.7	(0.74)	24
	5.01	10	3.4	3.7	(0.76)	21
	6.04	60	3.0	1.8	(0.91)	75
Benzoic acid pK : 4.2	3.02	1	0.94	28	(0.64)	28
	4.03	4	2.4	16	(0.83)	51
	5.03	20	2.8	5.2	(0.72)	46
	6.02	100	2.0	2.3	(0.75)	73
Salicylic acid	3.04	2	1.0	49	(0.61)	75
	4.02	10	0.90	16	(0.73)	86
	5.00	100	1.0	3.2	(0.70)	81
	6.01	>150	—	1.5	(0.91)	—

Koji extract (Ballg. 7°) containing an antiseptic was inoculated with washed bakers' yeast in 0.0003% and incubated for 48 hours at 30°C. The concentration of the undissociated antiseptic was calculated from the ionization constant. Values of k and n were obtained in the buffer solution at 30°C.

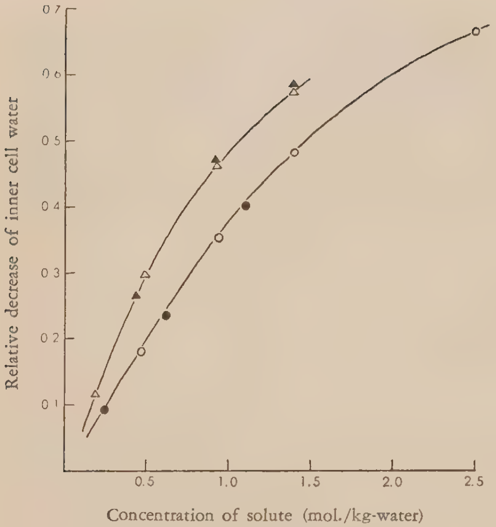

FIG. 1. Relative Decrease of Inner Cell Water of Yeast by Physical Action of Osmotic Pressure

Bakers' yeast were suspended in the solution of various concentration of the solutes for one hour. Then, the ratio of the abstracted water to the original water content of 0.65 (kg/kg-cell) in the yeast cells was evaluated from the decrease of the centrifuged volume of the cells.

○: glycerin, ●: galactose,
△: potassium chloride, ▲: sodium chloride.

in the same table are almost independent of the pH value. This fact supports the previous assumption that the pH in the medium affects the transfer ratio of the antiseptics and, accordingly, it results in the variation of the toxic effect.

Influence of glycerin on transfer. In the concentrated solution of glycerin, the water in the yeast cell is abstracted by the physical action of the osmotic pressure as seen in Fig. 1.

The transfer ratio of the antiseptics is also reduced with the concentration of glycerin as seen in Fig. 2. From this fact, some part of the transferred quantity of the antiseptics seems to change with the water content in the cell, and the quantity which is a function of the water content is considered as the dissolved quantity in the inner cell fluid.

Supposing that the concentration of the dissolved antiseptics in the inner cell fluid is in-

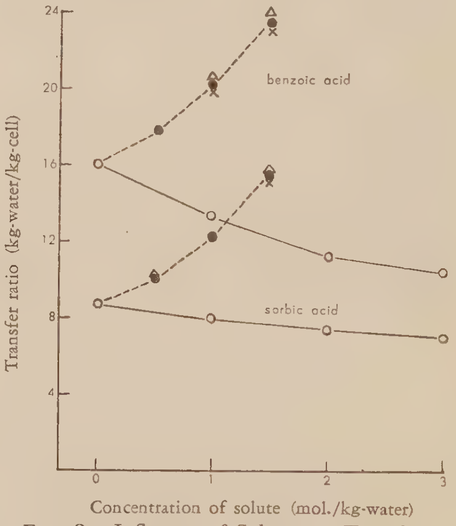

FIG. 2. Influence of Solutes on Transfer Ratio of Acid Antiseptics

Five percent of yeast cells were suspended in the buffer solution containing 2 m-mol./kg-water of an antiseptic and a certain quantity of glycerin or inorganic salts at pH 4.0±0.03. The mixture was let to stand for one hour at 30°C, and then, the transfer ratio was determined.

○; glycerin, ●: potassium chloride,
△: sodium chloride, ×: sodium nitrate.

TABLE II. INFLUENCE OF GLYCERIN ON TRANSFER OF ACID ANTISEPTICS

Antiseptic	*k* (*n*)		Difference of *k* value	Dissolved quantity $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$	Adsorbed quantity $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$	Adsorbed quantity / Transferred quantity
	Original buffer	Glycerin added				
Dehydroacetic acid	8.3 (0.89)	7.3 (0.89)	1.0	1.7	6.6	0.80
Sorbic acid	8.7 (0.74)	7.2 (0.71)	1.5	2.5	6.2	0.71
Benzoic acid	16 (0.83)	10.8 (0.77)	5.2	8.7	7.3	0.46
Salicylic acid	16 (0.73)	8.7 (0.88)	7.3	12.2	3.8	0.24

Five percent of yeast cells were suspended in each buffer solution containing various concentration of antiseptics, and 2 mol./kg-water of glycerin was either added or not to each mixture. The mixtures were let to stand for one hour at pH 4.0±0.03 and 30°C, and the values of *k* and *n* were determined.

The relative decrease of water content of the cell was evaluated to be 0.60 from the result in Fig. 1.

dependent of the concentration of glycerin in the medium, the dissolved quantity of the antiseptics in the original state of the cell can be evaluted from equation (2),

$$a_d=(a-a_g)/w \qquad (2)$$

where a_d is the dissolved quantity, a the transferred quantity in the original state, a_g the transferred quantity when glycerin is added to the medium, and w the relative decrease of the water content of the cell due to the change of the osmotic pressure of the medium by the addition of glycerin.

In Table II, the values of k are the transferred quantity under the condition that the equilibrium concentration of the antiseptics is 1 millimole per kg of water in the medium. From equation (2), therefore, the dissolved quantity in the original state can be evaluated at the same equilibrium concentration by substituting the value of k in the original medium and that in the medium containing 2 moles of glycerin per kg of water in a and a_g, respectively. (In this case the corresponding value of w is given to be 0.60 in Fig. 1.) The evaluated dissolved quantities are given in Table II. Hence, the difference between the transferred quantity in

TABLE III. INFLUENCE OF SOME SOLUTES ON TOXIC EFFECT OF ACID ANTISEPTICS

	Conc. of solute (mol./kg-water)	Minimum inhibiting concentration (m-mol./kg-water)			
		Dehydroacetic acid	Sorbic acid	Benzoic acid	Salicylic acid
Control	0	0.5	4	4	10
Glycerin	2	0.5	4	4	10
Glucose	2	0.3	3	3	7
Potassium chloride	1	0.3	2	2	4
Sodium chloride	1	0.1	1	1	2
Sodium nitrate	1	0.1	1	1	2

The conditions were the same as in the experiment of Table I. Since in the case of the sodium salts the yeast growth was slow, the growth was observed after 120 hours of incubation (Fig. 3).

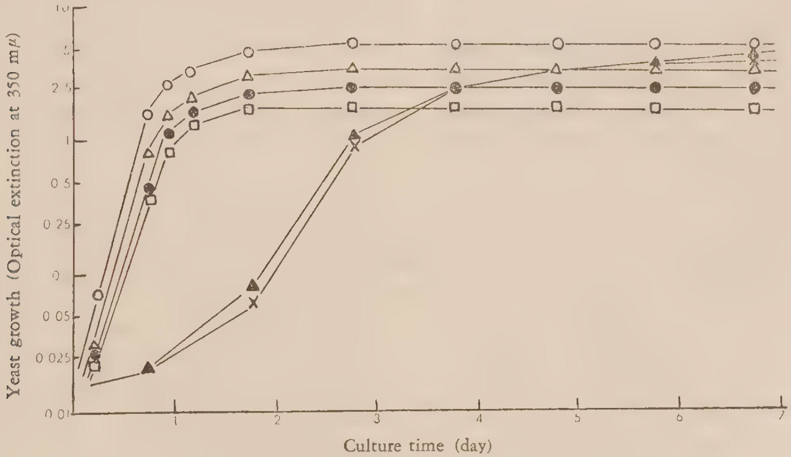

FIG. 3. Influence of Solutes on Yeast Growth

Koji extract (Ballg. 7°) containing 1 mol./kg-water of inorganic salts or 2 mol./kg-water of organic materials was inoculated with washed bakers' yeast in 0.01 percent and incubated at 30°C.

○: control, ●: glycerin, □: glucose, △: potassium chloride,
▲: sodium chloride, ×: sodium nitrate.

the original state and the dissolved quantity gives the quantity which is independent of the change of the water content in the inner cell fluid. The value of the difference is considered as the adsorbed quantity on the solid phase of the cell, and the ratio of the adsorbed quantity to the transferred quantity is given in the last column of Table II.

On the other hand, the inhibiting concentration of the antiseptics given in Table III scarcely changes by the addition of 2 moles of glycerin per kg of water in the medium, though glycerin increases the osmotic pressure of about 50 atm. and the dissolved quantity is reduced to one half of the original quantity.

In the case of glucose, the toxic effect increases as seen in the same table, but this result is considered to involve the inhibiting effect of the solute itself, because the yeast growth in the stationary phase is somewhat depressed by the addition of glucose as seen in Fig. 3.

Accordingly, it is considered that the toxic effect of the antiseptics is independent of the change of the dissolved quantity.

Increase of toxic effect by addition of inorganic salts. The addition of 1 mole of inorganic salts per kg water in the medium reduces the inhibiting concentration of the antiseptics as seen in Table III. Of course, the result with respect to the sodium salts may involve the inhibiting effect of the salts themselves, because the yeast growth in the induction phase is strongly depressed by the presence of the salts (Fig. 3). However, even in the case of potassium chloride, the inhibiting concentration is reduced to one half of the original concentration, though the influence of the salt on the yeast growth is smaller than in the case of glycerin (Fig. 3). Therefore, it is considered that the toxic effect of the antiseptics increases by the presence of the inorganic salts in the medium.

In this connection, it was previously reported[4] that the toxic effect of some antiseptics on fungi is more increased by the addition of the inorganic salts, being independent of the biological effect of the salts themselves. The present result on the yeast coincides with the previous result on fungi.

Influence of inorganic salts on transfer. In the concentrated solution of the inorganic salts, the inner cell water is similarly abstracted by the osmotic pressure as in the case of glycerin (Fig. 1). However, the transfer ratio increases with the concentration of the salts and this effect is essentially the same in any sort of salts used, as shown in Fig. 2.

From this fact, it is considered that the adsorbed quantity is increased by the addition of the inorganic salts in the medium. In this case, the concentration of the dissolved antiseptics in the inner cell fluid will increase with the increase of the adsorbed quantity, because the concentration of the dissolved antiseptic in the cell fluid is in the equilibrium with the adsorbed quantity. Accordingly, the relative increase of the adsorbed quantity, s, which is independent of the osmotic pressure can be given by eqnation(3),

$$s=(a_s/(a_a+a_d\,(1-w)))-1 \qquad (3)$$

where, a_a and a_d are the adsorbed quantity and the dissolved quantity in the original state, respectively, and a_s, the transferred quantity in the medium containing the salts, and w, the relative decrease of the inner cell water due to the change of the osmotic pressure of the medium by the addion of the salts.

In Table IV, the value of k is the transferred quantity under the condition that the equilibrium concentration of the antiseptic is 1 millimole and the concentration of potassium chloride is 1 mole per kg of water in the medium. From equation (3), therefore, the relative increase of the adsorbed quantity by the addition of 1 mole of potassium chloride per kg of water in the medium can be evaluated by substituting the values of k given in Table IV in a_s. (In this case the corresponding values of a_a, a_d and w have been given in Table II and Fig. 1.) The evaluated values of s given in the last column of Table IV are in the range from 0.54 to 0.99. This result essentially corresponds with the fact

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[4] S. Oka, *Annual Report of Food Industrial Experiment Station, Hiroshima Prefecture*, 5, 23 (1958).

TABLE IV. INFLUENCE OF POTASSIUM CHLORIDE ON TRANSFER OF ACID ANTISEPTICS

Antiseptic	k	(n)	Increase of k value	Relative increase of adsorbed quantity
Dehydroacetic acid	11.5	(0.92)	3.2	0.54
Sorbic acid	12.1	(0.71)	3.4	0.61
Benzoic acid	20.5	(0.91)	4.3	0.74
Salicylic acid	20.2	(0.56)	4.2	0.99

The conditions were the same as in the experiment of Table II, except the absence of glycerin and the presence of 1 mol./kg-water of potassium chloride in the medium. The relative decrease of the water content of the yeast cell was evaluated to be 0.48 from the result of Fig. 1.

that the toxic effect is increased about two times greater than the original effect by the addition of 1 mole of potassium chloride per kg of water in the medium (Table III).

In this case, the increase of the adsorbed quantity may depend upon the decrease of the ionization constant of the antiseptics owing to the increase of the ionic strength in the medium, because it results in the increase of the concentration of the undissociated antiseptics in the medium. However, the adsorption of the undissociated molecules of the antiseptics on the cell seems to be pronounced by the presence of the salts, because the thermodynamic activity of the lipophyl molecules is increased by the addition of the inorganic salts.

As mentioned above, in any condition of the medium, the toxic effect seems to depend more closely upon the adsorbed quantity than the total transferred quantity. Therefore, it is concluded that the adsorbed quantity of the acid antiseptics is the limiting factor of their toxic effect.

The adsorbed quantity required to inhibit the yeast growth can be evaluated from the transferred quantity under the inhibiting condition (Table I) and from the ratio of the adsorbed quantity to the transferred quantity (Table II) as given in Table V.

TABLE V. ADSORBED QUANTITY OF ACID ANTISEPTICS REQUIRED TO INHIBIT YEAST GROWTH

Antiseptic	Adsorbed quantity (m-mol./kg-cell)
Dehydroacetic acic	3.6
Sorbic acid	17
Benzoic acid	24
Salicylic acid	21

Acknowledgements. The author wishes to express his sincere thanks to Emeritus Prof. R. Sasaki and Prof. Y. Sakurai of Tokyo University for their interest in his work, and also to Dr. C. Hata, President of this institute, for his support and encouragement. The auther is greatly indebted to Dr. M. Fujimaki, Dr. S. Okimasu, Dr. R. Nōmi and Mr. K. Shimizu for their kind advice.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 344~350, 1960]

Fractionation of Sugar Phosphates and Nucleotides in Potato Tubers by Ion-exchange Chromatography*

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Phosphate compounds in potato tubers were fractionated by ion-exchange chromatography. By the use of radio-phosphorus, the following compounds were tentatively identified to be present in potato tubers: G-1-P, G-6-P, F-6-P, FDP, PGA, AMP, UMP, ADP, UDPX, UDPG and ATP. When the tubers were stored at a low temperature, the contents of organic phosphate compounds showed a considerable increase over those of potatoes stored at a high temperature. However, the relative composition of each component was not much influenced by the storage temperature except ATP which showed a great increase at a lower temperature.

It is well established that sugar contents of potatoes stored at a low temperature are greater than those of potatoes stored at a high temperature[1~3]. It has been reported that the contents of glucose, fructose and especially sucrose increase, while the starch content decreases, when potato tubers are stored at a lower temperature; the increased amount of sugars is approximately equal to the amount of starch disappeared. These results led Arreguin-Lozano and Bonner[1] to conclude that sugars are formed from starch. The results also suggest that there may be more or less changes in the contents of sugar phosphates and uridine and adenine nucleotides, which are known to play important rôles in the interconversion of sugars in plants. Concerning fractionation and analysis of sugar phosphates in potatoes as influenced by the temperature of storage, there has been a report of Arreguin-Lozano and Bonner[1], who claimed that there were remarkable changes in the contents of G-1-P**, G-6-P and F-6-P; i. e., G-6-P content was by far the greater at a higher temperature while the reverse was true for G-1-P and F-6-P. But they had made no attempt to analyze nucleotides, because the importance of uridine nucleotides in carbohydrate metabolism was not yet known at that time. Moreover, it has become gradually clear that the barium salt-alcohol fractionation method for phosphate compounds[4], which was employed by these authors for sugar phosphates, is not always suitable for the quantitative separation and analysis of phosphate compounds in biological materials[4,5]. Accordingly, it seemed interesting to reinvestigate the contents of sugar phosphates as well as to study the nucleotide contents in potatoes stored under different temperature conditions by the use of ion-exchange chromatography, which is, at the present time, generally believed to be the most suitable for the quantitative separation and analysis of phosphate compounds in biological materials[6,7].

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* The experimental data in this paper are taken from a thesis submitted by K. Mori in partial fulfilment of the requirements for the degree of master of agriculture in the University of Tokyo, 1959.

[1] B. Arreguin-Lozano and J. Bonner, *Plant Physiol.*, **24**, 720 (1949).

[2] S. Schwimmer, A. Bevenue, W. J. Weston and A.L. Potter, *J. Agr. Food Chem.*, **2**, 284 (1954).

[3] K. Mori and M. Nakamura, This Bulletin, **23**, 389 (1959).

** The following abbreviations are used throughout this paper: G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; FDP, fructose-1, 6-diphosphate; AMP, ADP and ATP, adenosine mono-, di- and triphosphate, respectively; UMP, uridine monophosphate; UDPAG, uridine diphosphate acetylglucosamine; UDPG, uridine diphosphate glucose; UDPX, unidentified uridine diphosphate sugar compound; PGA, phosphoglyceric acid; P*i*, inorganic orthophosphate.

[4] G.A. LePage, in W.W. Umbreit, R.H. Burris and J.F. Stauffer (eds.), Manometric techniques, p. 268. Burgess Publishing Co. (Minneapolis, 1957).

[5] T. Ohmura, *Nippon Nôgei-kagaku Kaishi* (*J. Agr. Chem. Soc. Japan*), **28**, 240 (1954).

However, as the acid or alcoholic extract of potatoes contained very large amounts of acid impurities in contrast to a small content of phosphate compounds, it was almost impossible to fractionate and determine the latter by the usual method of ion-exchange chromatograpy. After many unsuccessful trials, the method finally adopted was the one which utilized radiophosphorus to enhance the sensitivity of the analysis of phosphate compounds. By employing this procedure, the contents of sugar phosphates as well as nucleotides in potato tubers were estimated.

EXPERIMENTAL

Principle.

A strong solution of radioactive orthophosphoric acid was injected into potato tubers. After allowing these tubers to stand for several days at prescribed temperatures to reach equilibrium, a small amount of sample was cut off from the tuber and extracted with aqueous alcohol. A small amount each of several authentic specimens of phosphate compounds, the presence of which in potato tubers were suspected, was added to the extract previously freed of alcohol. The solution thus obtained was chromatographed on a Dowex-1 column. Both chemical and physical identification of phosphate compounds and radioactivity measurement were made on fractionated eluates from the column. The presence of radioactivity accompanying a phosphate compound indicates the most probable existence of that compound in potato tubers.

Materials and Methods.

Radioactive Orthophosphoric Acid: Carrier-free $H_3{}^{32}PO_4$ was obtained from the Japan Radioisotope Association.

Sugar Phosphates and Nucleotides: G-1-P, dipotassium salt[8,9]; G-6-P, barium salt, prepared enzymically[10]; F-6-P, barium salt[11]; FDP, barium salt[12]; ATP, tetrasodium salt, crude, obtained from Schwarz Labs., Inc.; UDPG, sodium salt[13].

Methods of Analysis: Inorganic orthophosphate was determined by the method of Nakamura[14]. The phenol method of Dubois et al.[15] and the resorcinol method of Roe[16] were used for the determination of total sugar phosphates and fructose phosphates, respectively, by using authentic specimens as standards. Nucleotides were determined and tentatively identified by ultraviolet spectrophotometry[17] and chromatographic behaviors. Radioactivity measurements were carried out with a Geiger-Müller counter attached to an Aloka decimal scaler.

Incubation of Potato Tubers: Three holes (approximately 1 cm deep) were cut from the surface into a potato tuber (*Solanum tuberosum* L. var. Irish cobbler) with a cork borer (diam. ca. 0.5 cm.). About 0.4 ml of a solution containing approximately 0.3 mC. of carrier-free radioactive orthophosphoric acid was put into the holes, in which were then inserted potato disks cut out previously into the cork borer and they were sealed with Scotch tape. Then, the tubers were incubated either at 30°C for 4.5 days or at 6°C for 9 days.

Preparation of the Sample Solution for Coromatography: After the incubation, the potato tubers were cut into pieces and 10∼15 g of tissues adjacent to the holes were taken for analysis, the surface of the holes being removed beforehand. Ethanol was added to the tissues to a final concentration of 80% and the homogenized suspension was refluxed on a water-bath in order to facilitate extraction of phosphate compounds and to inactivate phosphatases. The residue was again extracted two or three times with hot 50% ethanol and finally with cold 20% ethanol[6]. The combined extracts were concentrated in vacuo to about 5 ml and polysaccharides were removed by high-speed centrifugation. The latter procedure was essential to ensure a good chromatographic separation. Ten to 20 mg each of authentic phosphate compounds were added to the solution. Removal of cations and dissolution of added phosphate compounds were performed by shaking the solution with a small amount of Amberlite IR-112 (H^+) in the cold.

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TABLE I. EXTRACTION OF RADIO-PHOSPHORUS INJECTED INTO THE POTATO TUBERS.

Temperature of Storage	6°C		30°C	
Amount of Sample	15 g		10 g	
Extracting Agent	Volume	Total Radioactivity	Volume	Total Radioactivity
80% Ethanol	65 ml	344×10⁴cpm	42 ml	206×10⁴ cpm
50% 〃	35	570	26	312
50% 〃	35	138	23	113
50% 〃	34	64	—	—
20% 〃	36	10	20	28
Total	205	1126	111	659

Then, the pH of the solution was adjusted to 8.5 with diluted ammonia (total volume, 25∼30 ml).

Ion-exchange Chromatography: The solution was passed through a column of Dowex-1 chloride, 0.87 sq. cm×11 cm, 200∼400 mesh, at a flow rate of 0.5 ml/min. Free sugars were removed with 200 ml of 0.001 M aqueous ammonia. Phosphate compounds were eluted successively according to the procedure of Khym and Cohn[18] (flow rate, 1 ml/min.). The effluent solution, fractionated into 7-ml portions, was assayed for P*i*, sugar phosphates, nucleotides and radioactivity.

RESULTS

Extraction of Phosphate Compounds from Potato Tubers: The amount of phosphorus which could be extracted by aqueous ethanol was 0.02∼0.03% of fresh weight of potato tubers. The extraction pattern with successive portions of aqueous ethanol is shown in Table I. Approximately one-half or more of radio-phosphorus in the tubers remained in the residue (representing most probably phosphoproteins and nucleic acids). Total and inorganic phosphorus in the combined extracts were determined in order to enable calculations of the content of any phosphate compound in the tubers by the help of radioactivity data of that compound in question after chromatographic separation. In order to know the ratio of inorganic to organic radiophosphorus in the combined extracts, it was attempted to measure the radioactivity of the former after converting it to phosphomolybdic acid followed by extraction with isobutanol[19]. However, the content of P*i* thus obtained was always smaller than that obtained either by direct colorimetry or after chromatographic separation. The isobutanol extraction method seemed to be unsuitable for the determination of P*i* in the presence of a large amount of pectin-like substances.

Ion-exchange Chromatography of Phosphate Compounds: The elution pattern of phosphate compounds is shown in Fig. 1. Table II shows the total radioactivity corrected for the decay and the molar ratio of each component; the molar ratio was arbitrarily expressed by assuming UDPG to be 100. These results indicate that there may exist G-1-P, G-6-P, F-6-P, FDP, PGA, AMP, ADP, ATP, UMP, UDPG, UDPX (most probably UDPAG) besides P*i*. Although authentic specimens of AMP, ADP, UMP, and UDPAG were not added as such to the sample solution, these were present in amounts sufficient to permit detection by ultraviolet spectrophotometry. It may be most probable to assume that the former two were contained in the ATP sample and the latter two contained in the UDPG sample as contaminants, respectively. A peak which appeared just before that of ADP was assumed to be PGA from the chromatographic behavior in the solvent systems used. The content of organic phosphate increased by the low temperature treatment; a remarkable increase was observed in the contents of adenine nucleotides. There was no considerable change in the content of UDPG. Concerning sugar phosphates, the observation of Arreguin-Lozano and Bonner could not be confirmed; G-6-P was

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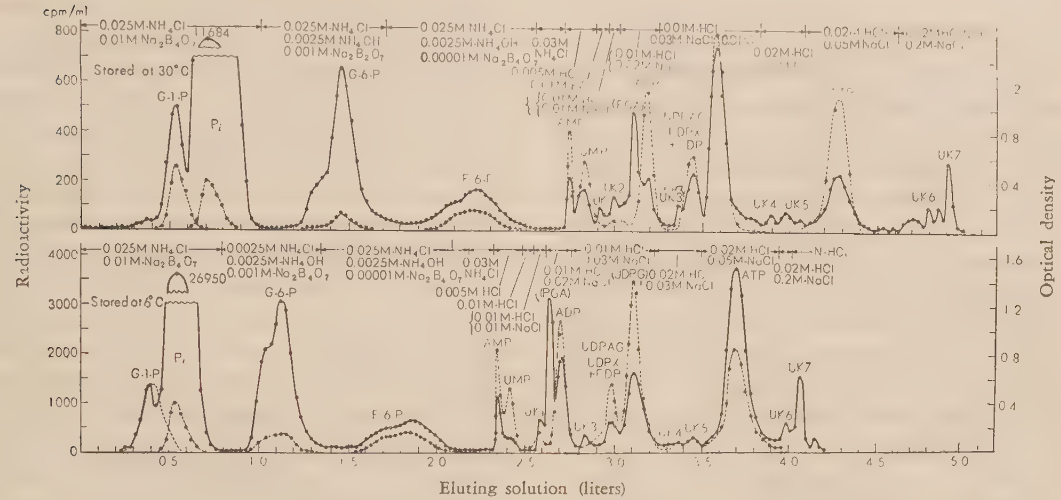

FIG. 1. Separation of Sugar Phosphates and Nucleotides in Potato Tubers by Ion-exchange Chromatography

See the text for experimental details. Solid line: radioactivity. Broken line: concentration of each compound (expressed as optical density of the solution in the appropriate assay system).

TABLE II. DISTRIBUTION OF RADIO-PHOSPHORUS IN VARIOUS PHOSPHATE COMPOUNDS IN POTATO TUBERS.

Condition of storage	6°C for 9 days		30°C for 4.5 days	
Phosphorus extracted with aq. ethanol	0.022%		0.024%	
	Radioactivity	Molar ratio	Radioactivity	Molar ratio
	cpm		cpm	
Total	5 695 300		2 173 700	
Pi	3 252 000	3151.1	1 615 500	3496.8
Sugar phosphates	1 024 600		279 900	
PGA	118 500	114.7	30 200	65.4
G–1–P	90 200	87.4	61 600	133.3
G–6–P	573 300	555.5	128 400	278.9
F–6–P	242 600	235.1	59 700	129.2
Nucleotides	1 418 700		278 300	
AMP	73 000	70.6	8 500	18.4
UMP	38 200	37.0	16 500	35.7
ADP	130 400	63.2	15 900	18.4
FDP+UDPX	79 100	38.4	22 900	24.9
UDPG	206 300	**100.0**	92 300	**100.0**
ATP	619 500	200.1	47 900	33.8
Unknowns	272 200		74 300	
UK 1	} 43 700		5 800	
UK 2			11 700	
UK 3	29 000		5 800	
UK 4	24 000		6 000	
UK 5	29 300		8 500	
UK 6	44 500		8 700	
UK 7	101 700		27 800	

always present more abundantly than F-6-P regardless of the temperature of storage.

With this eluting system, UDPX and FDP were eluted almost simultaneously, hence it was impossible to separate them. But approximate values for the two compounds were obtained by the following procedure (cf. Roe and Papadopoulos[20]): FDP was precipitated from the effluent solution in the presence of added P*i* with barium acetate. The radioactivity of the supernatant (due to UDPX) was compared with that of the original solution (FDP+UDPX). The results showed that FDP was eluted slightly after UDPX and they were approximately equal in amount.

DISCUSSION

Analytical Methods: Arreguin-Lozano and Bonner[1] reported that the content of sugar phosphates in potato tubers was 3.5~5.0% of dry weight when the analysis was carried out according to the barium salt-ethanol fractionation method of LePage[4]. So it was attempted at first to obtain sugar phosphates by precipitating them with barium and ethanol from the perchloric acid extract of potatoes. Then, barium was removed and the decationized solution was chromatographed on a Dowex-1 column. But actually, the phosphorus content of potatoes we could obtain in Japan was far less than that of potatoes used by the American investigators (their data show that the content of sugar phosphate-P is about 0.1% of fresh weight, while the phosphorus content of our potatoes is 0.02~0.03%, of which more than half is P*i*). Moreover, a considerable amount of pectin-like polysaccharides was present in our potatoes. These acid polysaccharides were the major component of the barium precipitate and interfered with subsequent chromatography.

The following methods were tested for the fractionation of phosphate compounds, but all in vain.

a) Phosphate compounds along with acid polysaccharides were precipitated with barium from the perchloric acid extract. Barium salts of hexose monophosphates were extracted with water from the precipitate, since they are known to be fairly soluble in water (Tankó[21] obtained hexose monophosphates formed in a phosphorylation reaction with pea enzymes by employing this method). It was expected that subsequent ion-exchange chromatography would reveal the composition of hexose monophosphates in potato tubers. But this method failed on account of the following two reasons. Firstly, hexose monophosphates could not be extracted completely from the barium precipitate with water.

TABLE III. EXTRACTION OF RADIOACTIVE G–6–P FROM BARIUM SALT PRECIPITATES CONTAINING HEXOSE MOMOPHOSPHATES.

Procedure A: The perchloric acid extract obtained from 300 g of potatoes was concentrated in vacuo and to the concentrated solution was added $G-6-^{32}P$ (about 1.7 mg, 1.0×10^5 cpm). Water-insoluble (Fraction I) and water-soluble alcohol-insoluble (Fraction II) barium precipitates were formed according to the method of LePage[4]. Fraction II was extracted with water.

Procedure B: P*i* was removed from the perchloric acid extract (see above) and $G-6-^{32}P$ (about 2.0 mg, 1.2×10^5 cpm) was added to the concentrated filtrate. Ethanol was added to the solution to give a final concentration of 60% and the precipitate (Fraction III) was removed. The supernatant was treated with barium acetate and ethanol and the 80% ethanol-insoluble barium precipitate (Fraction IV) was extracted with water.

Procedure A Fraction	Amount g	Racioactivity cpm
I	5.1	28 800
II	3.4	
Aq. extract		43 200
Residue	0.8	19 600
Total		91 600
Procedure B	g	cpm
III	2.1	66 100
IV	3.9	
Aq. extract		15 800
Residue	1.8	32 600
Total		114 500

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Table III shows the results of two recovery experiments by using radioactive G-6-^{32}P. Secondly, polysaccharides were also dissolved in water and gave a viscous solution which was unsuitable for chromatography.

b) The perchloric acid extract was neutralized with a concentrated solution of potassium hydroxide and the resulting potassium perchlorate was removed by centrifugation in the cold. All acid substances present in the supernatant were adsorbed on a column of Amberlite IR-4B, acetate form. After washing the column with water, the acid substances were eluted with a sodium hydroxide solution and the phosphate compounds were precipitated as the barium salt, which, after removal of barium, was used for chromatography. The acid pectin-like substances behaved similarly as phosphate compounds in the adsorption and desorption processes. The presence of a large amount of acid impurities interfered with the adsorption of phosphate compounds on a Dowex-1 column.

c) Potatoes were extracted with aqueous ethanol and after the removal of ethanol, the solution was passed through a long column of Dowex-1 (2.0 sq. cm. × 35 cm) according to Goodman et al.[22] But phosphate compounds were not adsorbed completely on account of the presence of a large amount of acid impurities.

d) The barium salt precipitate obtained by the method b) was dissolved and the resulting solution was passed through a column of Dowex-1, borate form, which adsorbed not only all the phosphate compounds but also sugars and pectin-like polysaccharides. Sugars and polysaccharides were washed off from the column with a dilute sodium tetraborate solution, and then G-1-P, G-6-P and F-6-P were eluted according to the method of Goodman et al.[22] However, a good separation was not obtained. Moreover, G-6-P and nucleotides were eluted simultaneously by 0.2 N sodium chloride solution, because preliminary separation of sugar phosphates from the nucleotides was impossible in the case of potatoes (see method a)) and accordingly this procedure was omitted.

An additional difficulty in these methods mentioned above is that 2~3 kg of potatoes must be used in order to secure a sufficient amount of each phosphate compound to be identified by chemical and physical methods, because potatoes contain only a very small amount of these compounds.

Since the separation of phosphate compounds from the acid impurities was almost impossible, it seemed advantageous to carry out analysis with a small quantity of sample which contained only a small amount of impurities to disturb neither adsorption of phosphate compounds on nor subsequent elution of them from the column. For that purpose, it was necessary to employ radio-phosphorus which would enable us to detect ultramicro quantities of each phosphate compound. So a method was devised which has been described in the experimental section. As it has already been confirmed[3] that a remarkable change in sugar composition occurs within several days under different temperature conditions, it was assumed that the incorporation of radio-phosphorus into phosphate compounds participating in the carbohydrate metabolism could also occur within the same period. But it is not guaranteed that the incorporation of radio-phosphorus proceeds at the same rate for all the phosphate compounds. So, the results thus obtained does not always represent a completely quantitative data. Nevertheless, it is possible to detect the existing phosphate compounds qualitatively, and further, to make a relative comparison concerning the composition of phosphate compounds in potatoes stored either at a high or at a low temperature.

By employing this method, it may be possible to determine the composition of phosphate compounds in such materials as sweet potatoes, beans and cereals, of which phosphate fractionation has been quite difficult because of the presence of interfering polysaccharides. It may also be possible to fractionate phosphate compounds in microorganisms, of which sample is difficult to be obtained in a large quantity, although it is impossible to identify each compound unequivocally by this method alone.

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Relation between the Storage Temperature and the Contents of Phospate Componnds: Table IV shows the content of sugars[3)] and phosphate compounds in potatoes stored at a high or at a low temperature. Further details of the composition of sugar phosphates and nucleotides are shown in Table V. Concerning the content of phosphate compounds, the authors could not observe such a remarkable change as was observed in the content of sugars. This agrees with the well-known fact that the amount of an intermediate compound does not always increase even when the amount of a final product shows a remarkable increase. The presence of UDPG suggests that probably it also plays an important rôle in the biosynthesis of sucrose in potato tubers, just as is known for several plant materials (see a short review[23)] on this subject). Although the content of UDPG did not increase during the low temperature storage, its turnover-rate might have been accelerated owing to the increased amount of ATP. Anyway, the composition of phosphate compounds in fresh potato tubers was not much influenced by the temperature of storage. This is quite contrary to Arreguin-Lozano and Bonner[1)] who obtained their data on dried materials, employing another method of analysis.

TABLE IV. THE CONTENTS OF CARBOHYDRATES AND AQUEOUS ETHANOL-SOLUBLE PHOSPHATE COMPOUNDS IN POTATO TUBERS AS INFLUENCED BY THE TEMPERATURE OF STORAGE

Temperature of storage	6°C	30°C
Starch	12.0	11.3
Sugars	1.62	0.31
Sucrose	1.12	0.28
Fructose	0.25	0.005
Glucose	0.25	0.024
Total P soluble in aq. ethanol	0.022	0.024
P*i*	0.012	0.018
Sugar phosphate-P	0.004	0.003
Nucleotide-P	0.006	0.003

The figures in the table are given in percentage of fresh weight.

TABLE V. RELATIVE CONTENTS OF SUGAR PHOSPHATES AND NUCLEOTIDES IN POTATO TUBERS

Temperature of storage	6°C	30°C
Sugar phosphates	**100.0**	**100.0**
PGA	11.4	10.8
G-1-P	8.9	22.0
G-6-P	56.0	45.8
F-6-P	23.7	21.4
Nucleotides	**100.0**	**100.0**
AMP	5.0	3.0
UMP	2.5	6.0
ADP	9.9	5.7
UDPX+FDP	5.5	8.2
UDPG	14.5	33.2
ATP	43.5	17.2
Unidentified	19.1	26.7

Results are given in percentage of total sugar phosphates or total nucleotides, respectively.

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Microbiological Studies of *Coli-aerogenes* Bacteria*

Part XII. Occurrence of Glyoxylic Acid Reductase

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Isocitritase was found in *Achromobacter superficialis*, *Zygosaccharomyces soya* and *major* in addition to *coli-aerogenes*. The mode of degradation of citrate was investigated with both the cell-free extracts and the ammonium sulfate-precipitates of *coli-aerogenes*. The addition of TPN** to the reaction mixtures brought about a striking increase in the yield of α-ketoglutarate against the decreasing yield of glyoxylate.

Little or no formation of α-ketoglutarate occurred when incubations were carried out with citrate in the presence of a large amount of succinate. However, when glyoxylate was added to a citrate-containing medium, the anaerobic formation of α-ketoglutarate took place even under such conditions as in the presence of succinate. Glyoxylate was found to be reduced to glycollate by the bacterial preparations. *Pseudomonas fluorescens* was also observed to produce glycollate from citrate. It was demonstrated that the formation of α-ketoglutarate with citrate is the result of a coupling reaction between isocitric dehydrogenase and glyoxylic reductase.

INTRODUCTION

Recent studies carried out on the mode of metabolisms of C_6-tricarboxylic acids in the bacteria of *coli-aerogenes* have demonstrated that these organisms can effect a C_2 plus C_4 cleavage of citrate in addition to the formation of α-ketoglutarate by way of the Krebs cycle. The splitting reaction of citrate into C_2 and C_4 units proceeded as follows:

(1) Citrate $\rightleftharpoons$ isocitrate $\xrightarrow{\text{isocitritase}}$ glyoxylate +succinate[1~6)]

(2) Citrate $\xrightarrow{\text{citritase}}$ acetate+oxalacetate[5,7~11)]

In the present investigation, the authors' attention has been directed to glyoxylic acid which is formed from citrate according to Reaction 1. An enzyme, glyoxylic acid reductase which catalyzes the reduction of glyoxylic acid to glycollic acid has recently been found in plant tissues[12~14)].

It has moreover been ascertained that diphospho- and triphosphopyridine nucleotides serve as the coenzyme for glyoxylic acid reductase in plant tissues (Reaction 3)[14,15)].

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** The following abbreviations are used: TPN=triphosphopyridine nucleotide, TPN^+ and TPNH=oxidized and reduced triphosphopyridine nucleotide, respectively; DPN^+ and DPNH=oxidixed and reduced diphosphopyridine nucleotide, respectively; $m(\mu)M=(\mu)$ mole(s).

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3) H. Katagiri and T. Tochikura, This Bulletin, **22**, 143 (1958).
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$$(3)\quad \underset{\text{Glyoxylate}}{\text{CHO-COO}^-} + \text{DPNH (TPNH)} + \text{H}^+ \rightleftharpoons \underset{\text{Glycollate}}{\text{CH}_2\text{OH-COO}^-} + \text{DPN}^+ \ (\text{TPN}^+)$$

EXPERIMENTAL AND RESULTS

Microorganisms. The strains employed in this paper were: *Escherichia coli* (strain G-2); *Aerobacter aerogenes* (strain B-2); *Pseudomonas fluorescens* (strain 346); *Achromobacter superficialis* (strain A-S); *Zygosaccharomyces soya* (strain C-6); *Zygosaccharomyces major* (strain C-I).

Analytical Methods. Keto-acids were identified by chromatography of the 2,4-dinitrophenylhydrazones[16~18], and determined by the methods of Cavalini et al.[16] or Friedemann and Haugen[19]. The protein content of bacterial extracts was estimated by turbidity measurements of trichloroacetic acid-precipitates according to the method of Stadtman et al.[20]. Reduction or oxidation of triphosphopyridine nucleotide was determined spectrophotometrically at 340 mμ with a Beckman DU spectrophotometer, using 3-ml quartz cells, 1-cm light path.

Chemicals. Glyoxylate was prepared from the bisulfite addition compound following the method of Weinhouse and Friedmann, and contained sodium chloride[21]. Triphosphopyridine nucleotide was purchased from the Tokyo Kasei Kogyo Co. Ltd., Tokyo.

Distribution and Formation of Isocitritase.

It has already been ascertained with the bacteria of *coli-aerogenes* that isocitritase is contained in the various cells obtained with the following growth-media: bouillon-, peptone-, glucose plus ammonium nitrogen-, acetate plus bouillon-, succinate plus bouillon- and citrate plus bouillon- media[3~6]. In the present paper, experiments were carried out with other microorganisms such as *Achromobacter superficialis*, *Zygosaccharomyces soya* and *major*. *Achromobacter superficialis* was grown aerobically at 28°C for 1 day on a medium containing 2% peptone, 0.5% each yeast extract and NaCl, while *Zygosaccharomyces soya* and *major* grown aerobically at 30°C for 3 days on the media containing 4% malt extract, 0.5% each yeast extract and peptone, and 3～10% NaCl. After cultivation, the organisms were collected by a centrifuge, and washed with distilled water. The cells were disintegrated by treatment with a 10 kc Raytheon oscillator for 20 minutes in the same manner as previously reported[4,5]. The cell debris was removed by a centrifuge and the fresh cell extracts were used without dialysis. The results of experiments are shown in Table I, in which incubations were carried out anaerobically at 37°C for 4 hours in the media (7 ml) containing 500μ M phosphate buffer, pH 7.4, 20μ M $MgSO_4$, 200μ M Na-citrate and 2 ml fresh cellfree extracts (obtained from 50 mg cells). From the data shown in Table I, it has been found that isocitritase exists in microorganisms such as *Achromobacter* and *Zygosaccharomyces* strains.

TABLE I. DISTRIBUTION OF ISOCITRITASE AND ISOCITRIC DEHYDROGENASE

Microorganisms and strains	Glyoxylate formed (μM)	α-Ketoglutarate formed (μM)
Zygosaccharomyces soya, C-6	9.8	2.1
Zygosaccharomyces major, C-I	2.5	2.3
Achromobacter superficialis, A-S	12.5	2.0

Furthermore, the formation of α-ketoglutarate by *Zygosaccharomyces soya* or *major* has been ascertained to be catalyzed by a TPN-linked isocitric dehydrogenase.

Influence of the Addition of Triphosphopyridine Nucleotide and Succinate upon Degradation of Citrate by *Coli-aerogenes*. In the previous papers of this series, the following facts have been found: the presence of a large amount of Na-succinate does not produce any inhibiting effect upon the activity of citritase but brings about a complete inhibition on isocitritase[5]; (2) the addition of TPN to a reaction mixture containing citrate and the bacterial cell extract results in the increasing yield of α-ketoglutarate against the decreasing yield of glyoxylate[4,6]. In the present paper, the authors have investigated the influence of succinate and TPN upon the degradation of citrate by the bacteria of *coli-aerogenes*. Expeiments with the cell extract of *E. coli* (strain G-2) were at first carried out in the following manner. *E. coli* was grown on a shaker at 30°C for 20 hours in a medium containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, 5 mg% $FeSO_4 \cdot 7H_2O$ and 1% $CaCO_3$. The cell-free extract was prepared from sonically treated cells. The reaction mixtures (5 ml) contained 500μ M phosphate buffer, pH 7.5, 15μ M $MgSO_4$, 100μ M Na-citrate, 2 ml the fresh cell extract without dialysis (obtained from 60 mg cells) and the requisite amounts of Na-succinate and TPN; 4 hours' incubation at 37°C in a Thunberg tube (in vacuo). Experimental results are shown in Table II. From this it will be seen that the addition of TPN to the reaction mixture brings about a striking increase in the yield of α-ketoglutarate against the decreasing yield of glyoxylate. Similar experiments were carried out with *A. aerogenes* (strain B-2). The organism was grown aerobically at 30°C for 20 hours in a medium containing 2% Na-citrate and 1% bouillon. The results of experiments carried out with both fresh cell extracts and ammonium sulfate-precipitated enzymes are shown in Tables III and IV. Fractionation of the cell extracts with ammonium sulfate was carried out in the same manner as previously reported[5].

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Table III: the reaction mixture (15 ml) contained 1 mM phosphate buffer, pH 7.5, 60 μM $MgSO_4$, 200 μM Na-citrate, 3 ml the cell extract of *A. aerogenes* (obtained from 60 mg cells of strain B-2) and the requisite amounts of Na-succinate and TPN; 150 minutes' incubation anaerobically at 37°C. Table IV: The reaction mixture (10 ml) contained 1 mM phosphate buffer, pH 7.5, 60 μM $MgSO_4$, 2 μM glutathione, 200 μM Na-citrate, 5 ml the ammonium sulfate-precipitates (obtained from 80 mg cells of strain B-2) and the requisite amount of TPN; 150 minutes' incubation anaerobically at 37°C. The results given in Tables III and IV may be summarized as follows: (1) when the fresh cell extract of *A. aerogenes* (strain B-2) or the ammonium sulfate 0.35~0.60 saturation fraction is anaerobically incubated with citrate in the absence of added TPN, a considerable amount of glyoxylate is produced but other keto-acids are never obtained in a noticeable amount; (2) when the anaerobic fermentation of citrate by the cell extract or the salt 0.35~0.60 fraction occurs in the presence of added TPN, a large amount of α-ketoglutarate is produced, under which conditions the yield of glyoxylate decreases; (3) when the fresh cell extract is anaerobically incubated with citrate in the presence of a large amount of succinate, little or no formation of keto-acids (glyoxylate and α-ketoglutarate) occurs; (4) the addition of TPN to the reaction mixture results in an extremely low yield of α-ketoglutarate when a large amount of succinate is present. These observations may indicate that there is a certain relationship between the degradation of citrate to α-ketoglutarate and the metabolic reaction of glyoxylate. It should be remembered here that a high concentration of succinate strongly inhibits the action of isocitritase.

TABLE II. INFLUENCE OF ADDITION OF SUCCINATE AND TPN UPON DEGRADATION OF CITRATE BY CELL EXTRACT OF *E. COLI*, STRAIN G-2, GROWN AEROBICALLY ON GLUCOSE

Exp. No.	I	II	III
Succinate added (M/l)	8×10^{-2}	0	0
TPN added (γ/ml)	0	0	25
Glyoxylate found (μM)	Trace	5.3	Trace
α-Ketoglutarate found (μM)	Trace	1.0	12.0

TABLE III. INFLUENCE OF ADDITION OF SUCCINATE AND TPN UPON ANAEROBIC DEGRADATION OF CITRATE BY CELL EXTRACT OF *A. AEROGENES*, STRAIN B-2, GROWN AEROBICALLY ON CITRATE-BOUILLON

Exp. No.	I	II	III	IV
Succinate added (M/l)	0	0	6.7×10^{-2}	6.7×10^{-2}
TPN added (γ/ml)	0	15	0	15
Keto-acids found (μM)				
Pyruvate	Trace	Trace	Trace	Trace
Glyoxylate	29.3	0	0	0
α-Ketoglutarate	Trace	35.5	Trace	4.8

TABLE IV. INFLUENCE OF ADDITION OF TPN UPON ANAEROBIC DEGRADATION OF CITRATE BY SALT-PRECIPITATES OBTAINED FROM CITRATE-GROWN CELL OF STRAIN B-2

Ammonium sulfate saturation fraction	0—0.35		0.35—0.60	
TPN added (γ/ml)	0	15	0	15
Glyoxylate found (μM)	0	0	2.8	Trace
α-Ketoglutarate found (μM)	0	0	0	5.2

Coupling Reaction between Isocitric Dehydrogenase and Glyoxylic Reductase. Table V shows the results of experiments on the degradation of citrate carried out under either aerobic or anaerobic conditions. The reaction mixtures contained 1.5 mM phosphate buffer, pH 7.5, 60 μM $MgSO_4$, 200 μM Na-citrate, the requisite amount of TPN and 5 ml the fresh cell extract (obtained from 120 mg cells of strain B-2 of *A. aerogenes* grown aerobically on citrate); total volume 12 ml; incubation for 140 minutes at 30°C under indicated conditions. It was found that when citrate was added to the fresh cell extract (without dialysis) of *A. aerogenes* under either

TABLE V. AEROBIC AND ANAEROBIC DEGRADATION OF CITRATE BY CELL EXTRACT OF STRAIN B-2 OF *A. AEROGENES* GROWN AEROBICALLY ON CITRATE-BOUILLON

Conditions of incubation	Anaerobic		Aerobic	
TPN added (γ/ml)	0	28	0	28
Glyoxylate found (μM)	29.1	Trace	32.6	Trace
α-Ketoglutarate found (μM)	Trace	36.3	Trace	38.5

TABLE VI. INFLUENCE OF ADDITION-TIME OF SUCCINATE AND TPN UPON ANAEROBIC DEGRADATION OF CITRATE BY CELL EXTRACT OF STRAIN B-2 OF *A. Aerogenes* WITH CITRITASE AND ISOCITRITASE

Exp. No.	I	II	III
Additions after 150 minutes' preincubation (100μM citrate and 1 ml cell extract were also supplemented)			
Succinate (M/L)	0	0	3.0×10^{-2}
TPN (γ/ml)	0	28	28
Keto-acids found after 330 minutes' incubation			
Pyruvate (μM)	6.5	4.8	4.5
Glyoxylate (μM)	22.5	Trace	Trace
α-Ketoglutarate (μM)	3.5	30.4	16.5

anaerobic or aerobic conditions, keto-acids rapidly appeared; glyoxylate was obtained in the absence of added TPN, whereas, α-ketoglutarate increased against decreasing glyoxylate in the presence of added TPN. There was no noticeable difference between the yields of α-ketoglutarate under both aerobic and anaerobic conditions. Since the degradation of citrate to α-ketoglutarate is a dehydrogenation, and also since there was an increase in the amounts of α-ketoglutarate against a decrease in the amounts of glyoxylate to be observed, this formation of α-ketoglutarate must be the result of a dehydrogenation coupled with a reduction of glyoxylate. This conclusion has furthermore been ascertained from the following experiments. Table VI shows the influence of the addition-time of both succinate and TPN upon the anaerobic degradation of citrate by the cell extract of *A.aerogenes* (strain B-2). The cell extract used here was prepared from a cell with both citritase and isocitritase, according to the method mentioned in the previous paper[5]. At first, reaction mixtures containing 200 μM phosphate buffer, pH 7.5, 9 μM $MgSO_4$, 100 μM Na-citrate, 1.5 ml the cell extract without dialysis (obtained from 45 mg of strain B-2) and water to a final volume of 3 ml were anaerobically incubated at 37°C for 150 minutes, and thereafter, 1 ml of the cell extract, 100 μM citrate and the requisite amounts of Na-succinate and TPN as indicated in Table VI were added to the incubated mixtures and anaerobic fermentations were continued at 37°C for 180 minutes; the final volume of second incubation was 7 ml.

Succinate was added in order to inhibit a progressive formation of glyoxylate from citrate. From the data shown in Table VI, the following facts have been elucidated. Glyoxylate which had been accumulated in the medium by preincubation, was remarkably decreased when the incubations were continued in the presence of added TPN. There was a decreasing yield of glyoxylate against an increasing yield of α-ketoglutarate. When both succinate and TPN were added to the reaction medium after 150 minutes' preincubation, a considerable amount of α-ketoglutarate was produced by successive fermentation. However, the additon of TPN brought about a higher yield of α-ketoglutarate in the absence of succinate than that in its presence. It has thus been demonstrated that the formation of α-ketoglutarate is the result of a coupling reaction between isocitric dehydrogenase and glyoxylic reductase. The results of experiments mentioned above indicate that the presence of a large amount of succinate causes a deficiency of glyoxylate in the medium by inhibiting the action of isocitritase, so that a coupling reaction between the dehydrogenation of citrate to α-ketoglutarate and the reduction of glyoxylate to glycollate tends to be depressed under the conditions. On the other hand, the formation of pyruvate from citrate (by the action of citritase) was not inhibited by the presence of a large amount of succinate as previously reported[5].

Observations mentioned above were further ascertained by the following experiments with chemically synthesized glyoxylic acid. In this case experiments were carried out with the ammonium sulfate 0.35~0.60 saturation fraction (dialyzed for 20 hours against distilled water) of strain B-2 of *A. aerogenes* grown aerobically on a citrate-bouillon. The reaction mixture contained 1 mM phosphate buffer, pH 7.5, 40 μM $MgSO_4$, 600 μM Na-succinate, 200 μM Na-citrate, 2 ml the salt 0.35~0.60 saturation fraction (corresponding to 45 mg cells), the requisite amounts of Na-glyoxylate and TPN, and water to a final volume of 10 ml; incubation for 5 hours at 37°C anaerobically. From the results shown in Table VII, it can be recognized that when the salt-fraction was incubated with citrate in the presence of such a large amount of succinate as to inhibit completely the action of isocitritase, the dehydrogenation of citrate to

TABLE VII. INFLUENCE OF ADDITION OF SUCCINATE AND TPN UPON CONSUMPTION OF GLYOXYLATE AND CITRATE BY SALT 0.35~0.60 SATURATION FRACTION OBTAINED FROM CITRATE-GROWN CELL OF STRAIN B-2 OF *A. Aerogenes*

Exp. No.	I	II	III	IV
Glyoxylate added (μM)	20.0	20.0	20.0	0
TPN added (γ/ml)	0	5	10	10
Residual glyoxylate (μM)	15.0	4.3	Trace	Trace
α-Ketoglutarate formed (μM)	0	13.0	17.7	Trace

α-ketoglutarate occurred only under such conditions that both glyoxylate and TPN were provided.

Spectrophotometric Observation. Fig. 1 shows the enzymic reduction and oxidation of TPN in the presence of citrate and other additions. Experiments were carried out with the ammonium sulfate 0.35~0.60 saturation fraction which was prepared from a cell of strain B-2 of *A. aerogenes* grown aerobically on a citrate-bouillon in the same manner as already mentioned. The salt saturation (0.35~0.60) fraction used here was also ascertained by macro-experiments, to be capable of catalyzing the following reactions; the formation of glyoxylate and succinate from citrate in the absence of TPN; the formation of α-ketoglutarate from citrate in the presence of TPN. For Curve A, the reaction mixture contained 100 μM phosphate buffer, pH 7.3, 4 μM $MgSO_4$, 40 μM Na-citrate and 0.3 ml of the salt fraction. Approximately 0.15 μM TPN was added at zero time. After 3 minutes' incubation, 6 μM Na-glyoxylate and 200 μM Na-succinate were added. Curve B, the same as Curve A except that 6 μM Na-glyoxylate were added at zero time. The final volume was made up to 3.0 ml with water. Incubation, at room temperature. The readings were corrected for dilution after the additions. From the results shown in Fig. 1, it has been observed that the ammonium sulfate 0.35~0.60 saturation fraction catalyzes the reduction of TPN by citrate (isocitrate) and the oxidaton of TPNH by glyoxylate. It is of interest to note that a small amount of TPNH accumulates in the medium at an early stage of fermentation of citrate even under such conditions that isocitritase and TPN-linked glyoxylic acid reductase are present.

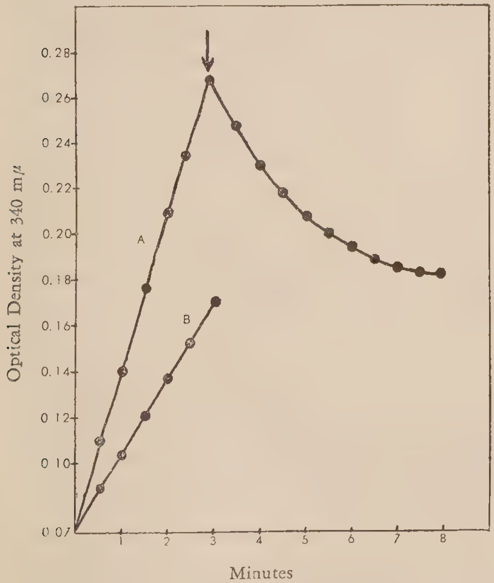

FIG. 1. Enzymic Reduction and Oxidation of TPN

⟶ glyoxylate plus succinate

TABLE VIII. FORMATION OF GLYOXYLATE, GLYCOLLATE, and α-KETOGLUTARATE FROM CITRATE BY CELL EXTRACT OF STRAIN B-2 OF *A. Aerogenes* GROWN ON CITRATE-BOUILLON

Exp. No.	I	II
TPN added (γ/ml)	0	25
Glyoxylate found (μM)	30.5	Trace
Glycollate found (μM)	0	25.0
α-Ketoglutarate found (μM)	0	38.5

Identification of Glycollic Acid. In order to ascertain the formation of glycollate from citrate, incubation was carried out anaerobically in the presence of added TPN (25γ/ml) at 37°C for 5 hours with a medium containing 1.5 mM phosphate buffer, pH 7.5, 20 μM $MgSO_4$, 7μM glutathione, 200 μM Na-citrate, 4 ml (obtained from 70 mg cells) of the dialyzed cell extract of strain B-2 of *A. aerogenes* grown on citrate, and water to a final volume of 10 ml. Another mixture without the addition of TPN was taken for sake of comparison. After incubation, the mixtures were deproteined with H_2SO_4 and then extracted with ether. The ether soluble fractions were spotted on sheets of filter paper (Toyo paper No. 51). Glycollic acid was separated on paper chromatograms with an alkaline-developing solvent of 8 volumes of ethanol, 1 volume of water and 1 volume of ammonium hydroxide[22]. A band on the paper corresponding to glycollic acid was cut out and the pieces of paper thus obtained, were eluted with a small amount of water. A colorimetric estimation of glycollic acid was carried out according to the method of Calkins[23]. The results of experiments are shown in Table VIII. It is evident that the anaerobic fermentation of citrate in the presence of TPN by the cell extract of *A. aerogenes* rise to a large amount of α-ketoglutarate and glycollate.

Another experiment was also carried out with the cell extract of strain 346 of *Pseudomonas fluorescens* grown aerobically for 20 hours at 28°C on the same citrate-bouillon medium as already mentioned. The results of experiments are shown in Table IX. The reaction mixtures contained 1 mM phosphate buffer, pH 7.5, 40 μM $MgSO_4$, 400 μM Na-citrate, 5 ml the cell extract (obtained from 80 mg cells), the requisite amount of TPN

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TABLE IX. ANAEROBIC DEGRADATION OF CITRATE BY CELL EXTRACT OF STRAIN 346 OF *Ps. Fluorescens* GROWN ON CITRATE-BOUILLON

Exp. No.	I	II
TPN added (γ/ml)	0	20
Glyoxylate found (μM)	85.5	42.5
Glycollate found (μM)	0	3.2
α-Ketoglutarate found (μM)	Trace	80.5

and water to a final volume of 15 ml; 4 hours' incubation anaerobically at 37°C. It has been found that glyoxylic acid reductase exists in the bacterial cell of *Ps. fluorescens* grown on a citrate-bouillon. However, in the case of *Ps. fluorescens* smaller amounts of glycollate were obtained than in the case of *A. aerogenes*.

Thus, it has been demonstrated that microorganisms such as *coli-aerogenes* and *Ps. fluorescens* contain glyoxylic acid reductase, and moreover a coupling reaction occurs in the presence of TPN as follows:

(1) $\text{Citrate} \rightleftharpoons \text{isocitrate} \xrightarrow{\text{isocitritase}} \text{glyoxylate} + \text{succinate}$

(2) $\text{Citrate} \rightleftharpoons \text{isocitrate} + \text{TPN}^+ \xrightarrow{\text{isocitric dehydrogenase}} \alpha\text{-ketoglutarate} + \text{TPNH} + \text{H}^+$

(3) $\text{Glyoxylate} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{glyoxylic reductase}} \text{glycollate} + \text{TPN}^+$

(4) $\text{Sum } 2\ \text{Citrate} \xrightarrow{\text{TPN}} \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{succinate} + \text{glycollate}$

TABLE X. INHIBITING EFFECT OF SEMICARBAZIDE ON FORMATION OF α-KETOGLUTARATE FROM CITRATE BY CELL EXTRACT OF STRAIN B-2 OF *A. Aerogenes*

Exp. No.	I	II	III
TPN added (γ/ml)	0	25	25
Semicarbazide added (M/L)	0	0	8×10^{-2}
Glyoxylate fohnd (μM)	35.7	Trace	62.5
α-Ketoglutarate found (μM)	0	50.5	Trace

Table X shows that semicarbazide (trapping agent) strongly inhibited the coupling reaction between isocitric dehydrogenase and glyoxylic reductase, when incubations were anaerobically carried out at 37°C for 5 hours with the media (10 ml) containing 1.5 mM phosphate buffer, pH 7.5, 20 μM $MgSO_4$, 7 μM glutathione, 200 μM Na-citrate, 4 ml the dialyzed cell extracts of *A. aerogenes* (obtained from 70 mg cells of strain B-2) and the requisites amounts of TPN and the inhibitor. This coupling reaction was also observed to be inhibited by KCN (5×10^{-3} M/l) or *p*-chloromercuribenzoate (1.5×10^{-4} M/l). However, *o*-phenanthroline (2×10^{-3} M/l), α,α′-dipyridyl (4×10^{-3} M/l) and quinacrine (1.7×10^{-3} M/l) were not effective. The anaerobic degradation of citrate to α-ketoglutarate by the cell extract of strain G-2 of *E. coli* was not inhibited by the antibiotics (400 γ/ml) including dihydrostreptomycin, chloramphenicol and tetracycline.

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Microbiological Studies of *Coli-aerogenes* Bacteria*

Part XIII. The Enzymic Oxidation of Glycollic Acid to Glyoxylic Acid

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The oxidation of glycollate to glyoxylate by molecular oxygen was found to be catalyzed by the dried cells of *coli-aerogenes* bacteria such as *E. coli* and *A. aerogenes*, and baker's yeast. The enzyme (glycollic oxidase) was ascertained to be contained in the bacterial cells of *coli-aerogenes* grown aerobically on various media such as glucose-ammonium salt, acetate-bouillon and citrate-bouillon. The bacterial cells with citritase and isocitritase were also found to contain glycollic acid oxidase. The cell-free extracts of *coli-aerogenes* were fractionated with ammonium sulfate for glycollic acid oxidase. The enzyme was resolved to yield an inactive apoenzyme which could be reactivated by the addition of FMN** or FAD. The enzyme was found to be strongly inhibited by cyanide. It was suggested that a glyoxylate $\rightleftharpoons$ glycollate system may function in the respiration of microorganisms.

INTRODUCTION

In the previous papers, the authors have studied the oxidative fermentations by *coli-aerogenes* bacteria of carbohydrates and organic acids which belong to the terminal respiration, and have demonstrated that these bacteria are capable of inducing α-ketoglutaric acid-fermentation under aerobic conditions[1~6,10,11]. Further investigations on the bacterial dissimilation of C_6-tricarboxylic acids have led the authors to elucidate the physiological relationship among citritase, isocitritase, isocitric dehydrogenase and glutamic dehydrogenase[5,7~9]. In the course of investigations on the metabolism of C_2-acids in *coli-aerogenes* bacteria, it has also been found by the authors that an enzyme catalyzing the reduction of glyoxylic acid to glycollic acid by reduced pyridine nucleotides is contained in microorganisms[12]. The present paper deals with the oxidation of glycollic acid to glyoxylic acid by microorganisms such as *coli-aerogenes* and baker's yeast. The function of glyoxylate $\rightleftharpoons$ glycollate system in plant tissues has been extensively studied by several workers[13~18]. The enzymic oxidation of glycollate by mammalian tissues was also reported by Dohan[19] and Kun et al.[20,21].

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** The following abbreviations are used: FMN=riboflavin-5′-phosphate (flavin mononucleotide); FAD=flavin adenine dinucleotide; TPN (DPN)=tri (di) phosphopyridine nucleotides; TPN^+ and TPNH=oxidized and reduced triphosphopyridine nucleotide, respectively; DPN^+ and DPNH=oxidized and reduced diphosphopyridine nucleotide, respectively; m (μ) M=m (μ) mole (s).

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EXPERIMETAL AND RESULTS

Methods. Analytical procedure was performed by the methods mentioned in the previous paper[12].

Chemicals. Glycollic acid and riboflavin-5'-phosphate were purchaced from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); flavin adenine dinucleotide from Wako Pure Chem. Ind. Co., Ltd. (Osaka); baker's yeast from Oriental Yeast Co., Ltd. (Osaka).

Oxidation of Glycollate to Glyoxylate by Dried Cells of *Coli-aerogenes* Bacteria and Baker's Yeast. In the previous papers[7~9,12], the following important facts were reported. When citric acid was added to either cell-free extracts of *coli-aerogenes* or the ammonium sulfate-precipitated protein of the bacteria, the formation of three kinds of keto-acids including glyoxylic, pyruvic and α-ketoglutaric acids occurred under the anaerobic conditions. There was a progressive increase with time in the amounts of α-ketoglutarate and a decrease in the amounts of glyoxylate under such conditions that a large quantity of triphosphopyridine nucleotide was present. Finally, an enzyme which catalyzed the reduction of glyoxylate to glycollate by either reduced diphospho- or triphosphopyridine nucleotide was detected in the bacterial cells such as *E. coli*, *A. aerogenes* and *Ps. fluorescens*.

Thus, it is now of interest to ascertain whether or not glycollate would be oxidized by the bacteria of *coli-aerogenes*. Experiments were at first carried out with the dried cells of *A. aerogenes* (strain B-2). The organism was grown on a shaker for 1 day at 30°C in a medium containing 2% Na-citrate, 1% bouillon and 0.4% yeast extract, and harvested by a centrifuge. The washed cells were dried with an electric fan at room temperature for 4~5 hours. The reaction mixture contained 4 mM phosphate buffer, pH 7.4, 4 mM semicarbazide, the requisite amount of Na-glycollate, 200 mg dried cells of *A. aerogenes*, and water to a final volume of 40 ml; 4 hours' incubation on a shaker at 30°C (see Table I). It was observed that when the dried cells of *A. aerogenes* (strain B-2) were aerobically incubated with glycollate in the presence of semicarbazide, a considerable amount of a keto-acid appeared in the medium. This keto-acid was identified as glyoxylic acid by chromatographic behaviour of the 2,4-dinitrophenylhydrazone. Subsequently, the oxidation of glycollate to glyoxylate was investigated with other strains of *coli-aerogenes* and a commercial baker's yeast. Baker's yeast was dried in the same mannner as in the bacteria mentioned above. The bacterial cells used here were as follows: (1) strain G-2 of *E. coli* grown aerobically for 20 hours at 30°C on 1% each Na-acetate and bouillon; (2) the same strain of *E. coli.* with a high potency of inducing α-ketoglutarate-fermentation, grown on the same glucose-ammonia medium as previously reported[3~5]; (3) strain B-T of *coli-aerogenes* with a high activity of citritase, grown on a citrate medium in the same manner as previously reported[8].

TABLE I. OXIDATION OF GLYCOLLATE BY DRIED CELL OF STRAIN B-2 OF *A. Aerogenes* GROWN AEROBICALLY ON CITRATE

Vessel No.	I	II	III*
Glycollate added (μM)	0	1000.0	1000.0
Glyoxylate formed (μM)	0	13.5	0

* Incubation after heat-treatment for 5 minutes at 90°C

Reaction mixtures contained 4 mM phosphate buffer, pH 7.4, 4 mM semicarbazide, 1000 μM Na-glycollate, the stated amounts of dried cells, and water to a final volume of 40 ml; 3~5 hours' incubation on a shaker at 30°C. From the results shown in Table II, it has been found that not only the dried cells of *coli-aerogenes* grown on the organic acid-containing media but those grown on a glucose medium can also catalyze the oxidation of glycollate to glyoxylate by molecular oxygen. This oxidation was further ascertained to occur by a dried cell of baker's yeast. No oxidation of glycollate was ever observed with a heated cell (see Table I).

Oxidation of Glycollate to Glyoxylate by Cell-Free Extracts of *Coli-aerogenes*. A cell-free extract of *coli-aerogenes* was prepared from a sonically treated cell in the same manner as previously reported. The results of experiments with the dialyzed cell extract are shown in Table III, in which incubations were carried out on a shaker for 3~5 hours at 30°C with the media (15~30 ml) containing 1~3 mM phosphate buffer, pH 7.4, 1~2.8 mM semicarbazide, 200~400 μM Na-glycollate and 5 ml dialyzed cell extracts (obtained from 100 mg bacterial cells). It will be seen that the dialyzed cell extracts of *coli-aerogens* possess an ability to oxidize glycollate to glyoxylate. This may indicate that the oxidation of

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TABLE II. FORMATION OF GLYOXYLATE FROM GLYCOLLATE BY DRIED CELLS OF *Coli-Aerogenes* BACTERIA AND BAKER'S YEAST

Organisms	Strain G–2 of *E. coli*		Strain B–T of *coli-aerogenes*	Baker's yeast
	grown on acetate	grown on glucose	grown on citrate	
Dried cells used (mg)	180	120	300	1500
Time of incubation (hours)	4	5	5	3
Glyoxylate formed (μM)	20.9	9.5	27.6	21.7

TABLE III. OXIDATION OF GLYCOLLATE TO GLYOXYLATE BY DIALYZED CELL EXTRACT OF *Coli-Aerogenes*

Enzyme source	A cell ext. of *E. coli* (strain G–2) grown on acetate	A cell ext. of *A. aerogenes* (strain B–2) grown on citrate	A cell ext. of *coli-aerogenes* (strain B–T) with citritase
Reaction mixtures (ml)	30	20	15
Time of incubation (hours)	4	5	3
Glyoxylate formed (μM)	9.5	6.5	4.5

TABLE IV. OXIDATION OF GLYCOLLATE BY SALT-FRACTION OF CELL EXTRACT OF *Coli-Aerogenes*

Organisms used	Ammonium sulfate saturation fraction of cell extracts	Glyoxylate formed (μM)
Strain G–2 of	0 ∼0.35	1.8
E. coli grown on	0.3∼50.70	0
acetate	(corresponded to 60 mg cells)	(3 hours' incubation
Strain B–2 of	0 ∼0.40	1.9
A. aerogenes grown	0.40∼0.70	0
citrate	(corresponded to 100 mg cells)	(5 hours' incubation)

TABLE V. EFFECT OF ADDITION OF FMN ON OXIDATION OF GLYCOLLATE BY DIALYZED CELL EXTRACT AND SALT-PRECIPITATES OBTAINED FROM ACETATE-GROWN *E. Coli*

Enzyme preparations	FMN added (M/l)	Glyoxylate formed (μM)
3 ml dialyzed cell extract	0	5.3
(corresponded to 90 mg	0	0*
cells of strain G–2)	1×10^{-4}	5.4
3 ml ammonium sulfate saturation fraction		
(corresponded to 70 mg cells of strain G–2)		
0 ∼0.35	0	2.0
0 ∼0.35	1×10^{-4}	2.0
0.35∼0.70	0	0
0.35∼0.70	1×10^{-4}	0

* Incubation after heat-treatment for 5 minutes at 90°C.

Reaction mixture contained 1m M phosphate buffer, pH 7.4, 1 mM semicarbazide, 200μM Na-glycollate and the indicated amounts of enzyme preparations and FMN: total volume 10 ml; 3 hours' incubation on a shaker at 30°C.

glycollate to glyoxylate is catalyzed by an oxidase in which DPN or TPN is not involved. The formation of glyoxylate was never observed in the absence of molecular oxygen.

Ammonium Sulfate Fractionation of Cell Extract. The cell-free extracts of *coli-aerogenes* were fractionated for glycollic acid oxidase, procedures being carried out at 0∼5°C. Solid ammonium sulfate was added to the cell extracts to give the saturation concentration as indicated in Table IV, and the precipitates formed were collected with a centrifuge and dissolved in 0.05∼0.1 M phosphate buffer (pH 7.4). The ammonium sulfate fractions were dialyzed against distilled water at 5°C for 20∼40 hours. The results of experiments carried out with the salt-precipitates are given in Table IV, in which the reaction mixtures (10 ml) contained 1 mM phosphate buffer, pH 7.4, 1 mM semicarbazide, 200 μM Na-glycollate and 3 ml (corresponding to 60∼100 mg cells) enzyme of *coli-aerogenes*; 3∼5 hours' incubation at 30°C on a shaker. From the data given in Table IV, it has been found that the salt 0.35 or 0.40 saturation fraction prepared from the acetate-grown or the citrate-grown cells of *coli-aergoenes* reveals an activity of glycollic acid oxidase, whereas, the salt 0.35∼0.70 or 0.40∼0.70 saturation fraction reveals no activity of the oxidase.

Prosthetic Group of Glycollic Acid Oxidase. It has been demonstrated by Zelitch and Ochoa[16] that glycollic acid oxidase of plant tissues is a flavoprotein with FMN as the prosthetic group. On the other hand, Kun et al.[21] have reported that glycollic acid oxidase of rat liver is resolved to yield an inactive apoenzyme which can be reactivated by FMN or FAD. These findings led the authors to clarify the prosthetic group of glycollic acid oxidase of microorganisms. Table V shows the effect of addition of FMN to the dialyzed cell extract or the salt-precipitated protein of *coli-aerogenes*. Experiments were carried out in the same manner as mentioned above, except the addition of flavin nucleotide. It will be seen that the addition of FMN revealed no striking effect on the oxidation of glycollate by the dialyzed cell extract or the salt-precipitates. However, these enzyme preparations were soon found to be resolved by the method of Warburg and Christian[22]. An inactive apoenzyme was obtained from the ammonium sulfate 0.35 or the 0.40 saturation fraction of the cell extract of *E. coli* grown aerobically on the acetate-bouillon, according to the following manner. The salt-precipitates were again suspended in a 0.6 saturation solution of ammonium sulfate at 0°C. The suspension was acidified with 2 N HCl to pH 2.3~2.5. After stirring for 60~120 minutes at 0°C, the precipitated protein was centrifuged and the supernatant fluid discarded. The precipitates were washed with a saturated ammonium sulfate and dissolved in 0.05 M phosphate buffer (pH 7.4). The reaction mixture contained 600 μM phosphate buffer, pH 7.0, 600 μM semicarbazide, 500 μM Na-glycollate, the requisite amounts of flavin nucleotides and 5 ml apoenzyme; total volume 10 ml; 3 hours' incubation at 30°C on a shaker. From the results of experiments given in Table VI, it has been found that the acid-treated enzyme can not catalyze the oxidation of glycollate to glyoxylate unless FMN or FAD is added. The reactivation of this apoenzyme in the presence of varying amounts of FMN is also shown in Table VII. The results of experiments indicated that the K_m for FMN was approximately 3×10^{-7} M.

TABLE VI. REACTIVATION OF GLYCOLLIC OXIDASE APOENZYME OF *E. Coli* BY FLAVIN NUCLEOTIDES

Flavin nucleotides added (M/l)		Glyoxylate formed (μM/10ml)
None		Trace
Riboflavin-5'-phosphate	5×10^{-5}	0.66
Flavin adenine dinucleotide	2.5×10^{-5}	0.63

TABLE VII. INFLUENCE OF CONCENTRATION OF ADDED RIBOFLAVIN PHOSPHATE UPON REACTIVATION OF GLYCOLLIC OXIDASE APOENZYME OF *E. Coli*

Riboflavin-5'-phosphate added (M/l)	Glyoxylate formed (μM/10 ml)
None	0.00
5.0×10^{-7}	0.50
2.5×10^{-6}	0.52
1.0×10^{-5}	0.53
1.0×10^{-4}	0.53

Experimental conditions, same as experiment shown in Table VI.

Inhibitors and Activator. Several substances were tested for their ability to accelerate or to inhibit the oxidation of glycollate by the enzyme of *coli-aerogenes*. Glutathione and $MgSO_4$ had no effect on the action of the salt-precipitated enzymes. No inhibiting effect was observed with α,α'-dipyridyl, 2,4-dinitrophenol, NaN_3 and quinacrine. However, cyanide has been found to be a powerful inhibitor. The results of experiments are shown in Tables VIII and IX. Table VIII: reaction mixture (10 ml) contained 500 μM phosphate buffer, pH 7.3, 1000 μM semicarbazide, 400 μM Na-glycollate, the requisite amounts of KCN and flavin nucleotides, and 4 ml of the salt 0.40 saturation fraction (obtained from 100 mg cells of acetate-grown *E. coli*); 4 hours' incubation at 30°C aerobically. Table IX: reaction mixture (10 ml) contained 500 μM phosphate buffer, pH 7.3, 500 μM semicarbazide, 500μ M Na-glycollate, requisite amounts of KCN and FMN, and 5 ml acid-treated enzyme (obtained from 80 mg bacterial protein of acetate-grown *E. coli*); 3 hours' incubation at 30°C aerobically. It will be seen that the addition of flavin nucleotides to the enzyme can not afford protection against cyanide. It appears that this oxidase may be a metalloflavoprotein.

TABLE IX. INHIBITING EFFECT OF CYANIDE ON OXIDATION OF GLYCOLLATE BY ACID-TREATED ENZYME OF *E. Coli*

KCN added (M/l)	None	None	5.0×10^{-3}
FMN added (M/l)	None	2.5×10^{-5}	2.5×10^{-5}
Glyoxylate formed (μM)	0.5	2.5	0.4

TABLE VIII. INHIBITING EFFECT OF CYANIDE ON OXIDATION OF GLYCOLLATE BY SALT 0.40 SATURATION FRACTION OF *E. Coli*

KCN added (M/l)	None	2.4×10^{-3}	2.4×10^{-3}	2.4×10^{-3}
Flavin nucleotides added (M/l)	None	None	FMN 3.2×10^{-4}	FAD 2.4×10^{-4}
Glyoxylate formed (μM)	4.1	1.2	1.2	1.5

DISCUSSION

Since two enzymes catalyzing glyoxylate $\rightleftharpoons$

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22) O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938).

glycollate system, i.e., glyoxylic acid reductase and glycollic acid oxidase are found in plant tissues, it has been considered that this system may play an important role in the respiration of green leaves[16~18; 23, 24]. In the presence of catalytic amounts of diphospho- or triphosphopyridine nucleotide and glyoxylic or glycollic acid, the glyoxylic reductase-glycollic oxidase system can act as a hydrogen transport system from reduced pyridine nucleotides to molecular oxygen.

In the previous papers[5, 7~9], the authors have demonstrated that when the intact or the treated cells of *coli-aerogenes* bacteria are incubated with citric acid under either aerobic or anaerobic conditions, little formation of α-ketoglutaric acid is observed, while these cells accumulate glyoxylic acid during the degradation of citric acid in the presence of an added trapping agent such as semicarbazide. It has moreover been found by the authors that microorganisms such as *coli-aerogenes* and *Ps. fluorescens* possess abilities to metabolize a certain part of citric acid according to the following reactions[12]. [A] Coupling reaction between isocitric acid dehydrogenase and glyoxylic acid reductase

(1) $\text{Citrate} \rightarrow \text{isocitrate} \xrightarrow{\text{isocitritase}} \text{glyoxylate} + \text{succinate}$

(2) $\text{Citrate} \rightarrow \text{isocitrate} + \text{TPN}^+ \xrightarrow{\text{isocitric dehydrogenase}} \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{TPNH} + \text{H}^+$

(3) $\text{Glyoxylate} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{glyoxylic reductase}} \text{glycollate} + \text{TPN}^+$

(4) $\text{Sum } 2\text{ Citrate} \xrightarrow{\text{TPN}} \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{succinate} + \text{glycollate}$

[B] Enzymic oxidation of TPNH and DPNH by glyoxylate

(5) $\underset{\text{glyoxylate}}{\text{CHO-COO}^-} + \text{TPNH (or DPNH)} + \text{H}^+ \rightleftharpoons \underset{\text{glycollate}}{\text{CH}_2\text{ OH-COO}^-} + \text{TPN}^+ \text{ (or DPN}^+\text{)}$

In the present paper, another enzyme, glycollic oxidase, which catalyzes the oxidation of glycollate by molecular oxygen has been further detected in microorganisms including *coli-aerogenes* and baker's yeast.

(6) $\text{Glycollate} \xrightarrow[\text{oxygen}]{\text{flavoprotein}} \text{glyoxylate}$

The combination of Reactions 5 and 6 provides an effective means of transferring hydrogen from reduced pyridine nucleotides to molecular oxygen.

$\text{Substrate} + \text{DPN}^+(\text{TPN}^+) \xrightarrow{\text{dehydrogenase}} \text{oxidation-product} + \text{DPNH (TPNH)} + \text{H}^+$

$\text{DPNH(TPNH)} + \text{H}^+ + \text{glyoxylate} \xrightarrow{\text{glyoxylic reductase}} \text{DPN}^+(\text{TPN}^+) + \text{glycollate}$

$\text{Glycollate} + \text{oxygen} \xrightarrow{\text{glycollic oxidase}} \text{glyoxylate}$

$\text{Sum Substrate} \xrightarrow{\text{oxygen}} \text{oxidation-product}$

Therefore, it has now been suggested that some part of respiration in microorganisms may proceed by a combined system of Reactions 5 and 6. However, it appears that the major reoxidation of reduced pyridine nucleotides may proceed by way of the diaphorase-cytochrome system rather than by way of glyoxylic reductase-glycollic oxidase.

The authors are greatly indebted to Dr. H. Chiba and Mr. F. Kawai for their helpful discussions of the problems.

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23) I. Zelitch, *J. Biol. Chem.*, **201**, 719 (1953).
24) I. Zelitch, *J. Biol. Chem.*, **216**, 553 (1955).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 362~371, 1960]

A New Basic Triple Salt Containing Magnesium Hydroxide

Part IV. The Quinary System KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O at 50°

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The quinary system KCl–K_2SO_4–$MgCl_2$–$MgSO_4$–$Mg(OH)_2$–H_2O and associated eight systems K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O, $MgCl_2$–$MgSO_4$–$Mg(OH)_2$–H_2O, KCl-$MgCl_2$–$Mg(OH)_2$–H_2O, KCl–K_2SO_4–$Mg(OH)_2$–H_2O, $MgSO_4$–$Mg(OH)_2$–H_2O, $MgCl_2$–$Mg(OH)_2$–H_2O, K_2SO_4–$Mg(OH)_2$–H_2O and KCl–$Mg(OH)_2$–H_2O were investigated at 50°. The solid phases of these systems were the new basic triple salt (NS salt B), $MgCl_2 \cdot 3Mg(OH)_2 \cdot 8H_2O$, $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$, carnallite, leonite, kieserite, hexahydrite, bischofite, potassium chloride, potassium sulfate and magnesium hydroxide and the crystallization fields of these salts in nine systems were determined.

INTRODUCTION

In the previous three papers[1~3], the 100° isotherms of this quinary system and associated eight systems were reported. Following these reports, the 50° isotherms of the same nine systems have been investigated in this study.

To date, a method to separate potassium dissolved in bittern directly as sulfate has never been established, so potassium in bittern has been separated as carnallite or potassium chloride. However, by the discovery of NS salt B and the accomplishment of the 100° isotherm of this quinary system, it has become possible to utilize potassium dissolved in bittern directly as sulfate. And it has also become possible to get a new fertilizer from bittern at 100°, because NS salt B is able to be used as a new basic potassium-magnesium fertilizer which contains potassium and magnesium both as sulfate and moreover magnesium hydroxide.

Though the crystallization field of NS salt B in this quinary system at 100° was very large, the yield of potassium as NS salt B from bittern was calculated from phase diagrams 50 per cent, because the amount of potassium dissolved in bittern was comparatively small.

Heretofore, the 50° isotherm of this quinary system has never been investigated, therefore in order to clarify the width of the crystallization field of NS salt B and the possibility of its separation from bittern and the yield of pottassium, if NS salt B were able to be separated from bittern, following nine systems have been investigated at 50°: the quinary system KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O and associated eight systems K_2SO_4-$MgSO_4$-$Mg(OH)_2$-H_2O, $MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O, KCl-$MgCl_2$-$Mg(OH)_2$-H_2O, KCl-K_2SO_4-$Mg(OH)_2$-H_2O, $MgSO_4$-$Mg(OH)_2$-H_2O, $MgCl_2$-$Mg(OH)_2$-H_2O, K_2SO_4-$Mg(OH)_2$-H_2O and KCl-$Mg(OH)_2$-H_2O.

RESULTS AND DISCUSSION

Experimental details were the same as described in the previous papers[1~3].

System KCl-$Mg(OH)_2$-H_2O at 50° There has been no report concerning this system.

Results obtained from this system are presented in Fig. 1. No double salt formation took place and nothing new resulted in this system. The saturated concentration of potas-

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[1] J. Sugi and M. Nakayama, This Bulletin, **22**, 208 (1958).
[2] M. Nakayama, *ibid.*, **23**, 46 (1959).
[3] M. Nakayama, *ibid.*, **24**, 147 (1960).

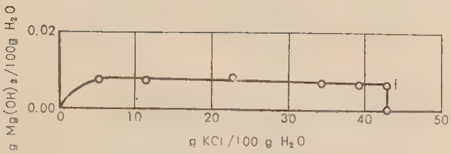

FIG. 1. The 50° Isotherm of the System KCl-$Mg(OH)_2$-H_2O.

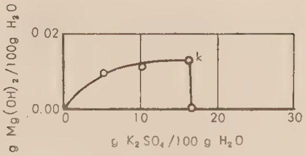

FIG. 2. The 50° Isotherm of the System K_2SO_4-$Mg(OH)_2$-H_2O.

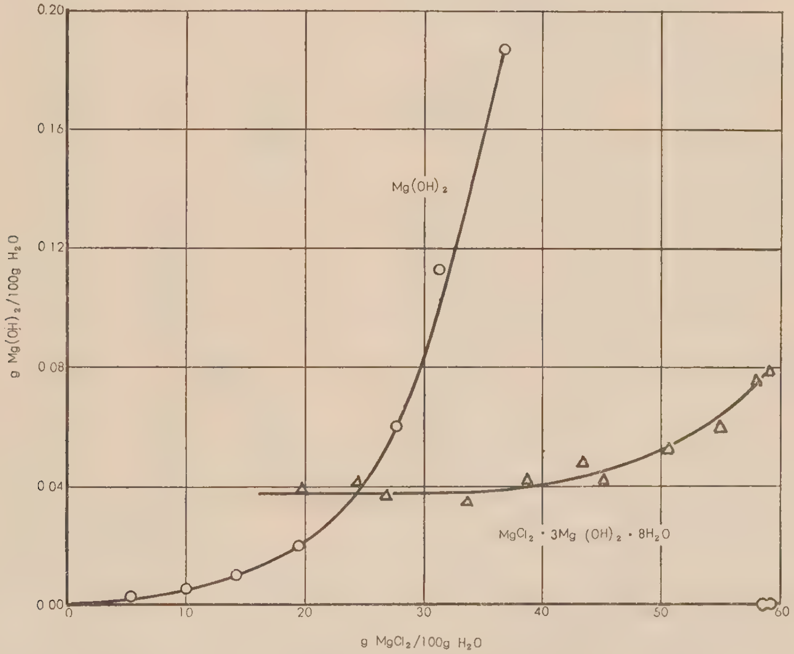

FIG. 3. The 50° Isotherm of the System $MgCl_2$-$Mg(OH)_2$-H_2O.

sium chloride agreed with those obtained on binary system KCl-H_2O[4,5].

System K_2SO_4-$Mg(OH)_2$-H_2O at 50° No report has been published on this system.

Results obtained from this system are presented in Fig. 2. No double salt formation took place and nothing new resulted in this system, too. The saturated concentration of potassium sulfate agreed with those obtained on binary system K_2SO_4-H_2O[6~8].

System $MgCl_2$-$Mg(OH)_2$-H_2O at 50° Concerning this system at 50°, one report was made by T. Maeda and S. Yamane[9]. According to their report, basic magnesium chloride $MgCl_2 \cdot 3Mg(OH)_2 \cdot 8H_2O$ was crystallized as the stable solid phase in such a solution as the concentration of magnesium chloride was higher than 16.29 gram per 100 gram of water. The solid phase in such a solution, as the concentration of magnesium chloride was lower than 16.29 gram

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[4] A.E. Hill and J.E. Ricci, *J.A.C.S.*, **53**, 4312 (1931).

[5] H. Hering, *Ann. Chim*, [11], **5**, 568 (1936).

[6] W. Schröder and H. Schackmann, *Z. anorg. Chem.*, **220**, 398 (1934).

[7] H. Hering, *Dissert Strasbourg*, **1926**, 50.

[8] E. Bovalini and E. Farbis, *Gazz.*, **65**, 619 (1935).

[9] T. Maeda and S. Yamane, *Bull. Inst. physic. Chem. Res.*, 1, 31 (1928).

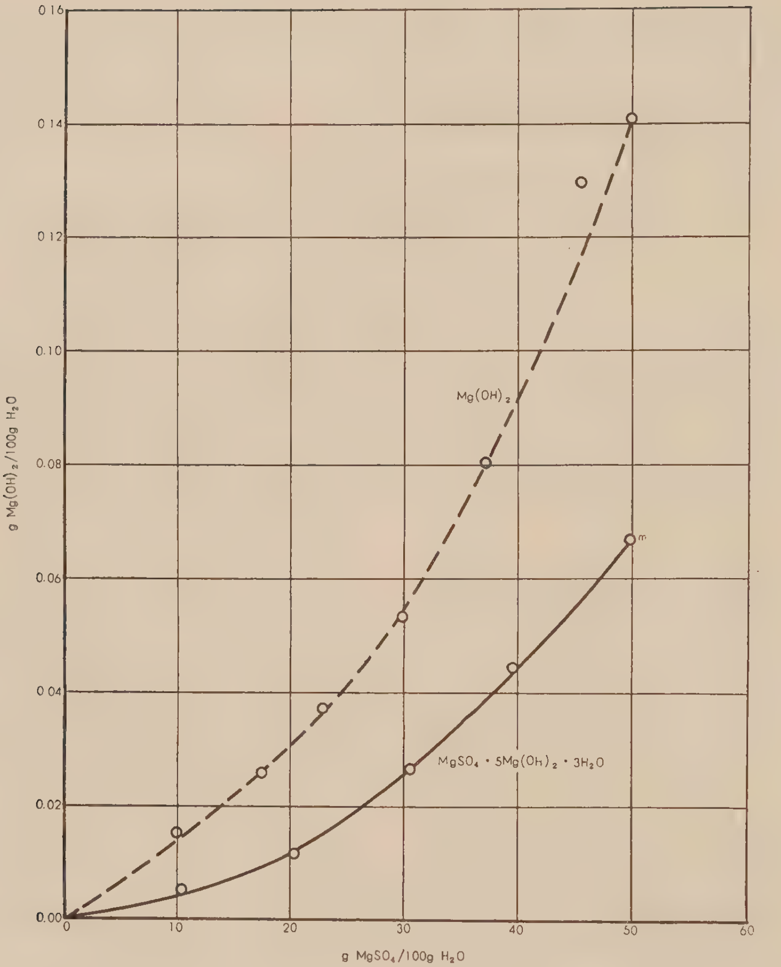

FIG. 4. The 50° Isotherm of the System $MgSO_4$-$Mg(OH)_2$-H_2O.

per 100 gram of water, was magnesium hydroxide.

Results obtained from this system are presented in Fig. 3. The chemical composition of double salt crystallized as the stable solid phase was the same as reported by T. Maeda and S. Yamane, but the concentration of magnesium chloride of transition point of this double salt was obtained to 24 gram per 100 gram of water as an intersection of solubility curves of magnesium hydroxide and basic magnesium chloride. This double salt may be existent as metastable solid phase in such a solution as the concentration of magnesium chloride is lower than the transition point, but finally it will be decomposed into magnesium hydroxide, the stable solid phase. The saturated concentration of bischofite agreed with those obtained on binary system $MgCl_2$-H_2O[10~12].

System $MgSO_4$-$Mg(OH)_2$-H_2O at 50° Heretofore

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[10] W. Biltz and E. Marcus, *Z. anorg. Chem.*, **71**, 169 (1911).
[11] A. Sieberts and H. Müller, *ibid.*, **189**, 249 (1930).
[12] J. Uhlig, *C. Min.*, **1913**, 419.

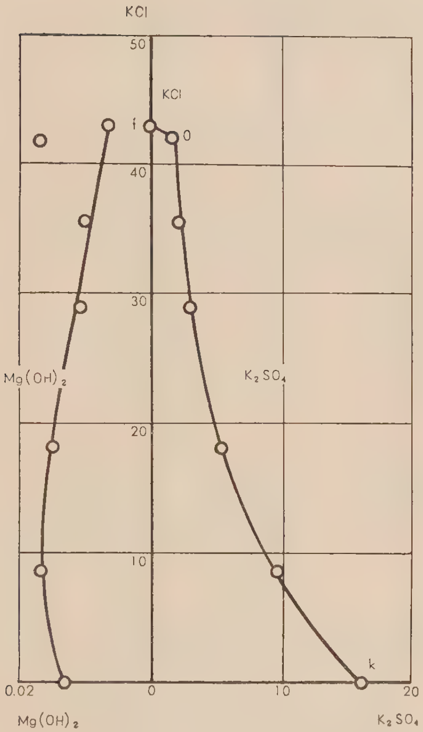

FIG. 5. The 50° Isotherm of the System KCl-K_2SO_4-$Mg(OH_2)$-H_2O [g salts/100 g H_2O].

though nothing has been reported on this system at 50°, in connection with the 80° isotherm, M-L. Delyon[13] reported that $MgSO_4 \cdot 3MgO \cdot 11H_2O$ was formed from the aqueous magnesium sulfate solution between the concentration of 300 and 500 gram per liter.

Results obtained from this system are presented graphically in Fig. 4. The stable solid phase of all regions was $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$, the same double salt as crystallized in the 100° isotherm of this system. This fact was confirmed by both powder X-ray diffraction and chemical analysis. At 50°, however, the formation of this double salt proceeded very slowly and it required two months or more to convent magnesium hydroxide to this double salt. The conversion speed depended upon the activity of added magnesium hydroxide.

The fact that the chemical composition of crystallized double salt was the same to the solid phase of 100° isotherm might be inconsistent with M-L. Delyon's conclusion, but our conclusion might not be mistaken because the composition of converted double salt was confirmed by both powder X-ray diffraction and chemical analysis and moreover it did not contradict to the solid phase of the system K_2SO_4-$MgSO_4$-$Mg(OH)_2$-H_2O at 50°.

The saturated concentration of hexahydrite coincide with those obtained on binary system $MgSO_4$-H_2O[14,15].

System KCl-K_2SO_4-$Mg(OH)_2$-H_2O at 50° Though there has never been any report concerning this system, concerning the 50° isotherm of ternary system KCl-K_2SO_4-H_2O, excluding magnesium hydroxide from the system, some papers have been published[16~18].

Results obtained from this system are presented in Fig. 5. The solubility of magnesium hydroxide in this system was so small that the diagram must be divided into two parts, namely those of potassium chloride-potassium sulfate and those of potassium chloride-magnesium hydroxide.

No double salt appeared from this system.

The chemical compositions of the liquid phase of monovariant point o, the co-existent point of three kinds of salts potassium chloride, potassium sulfate and magnesium hydroxide, were KCl, 42.07; K_2SO_4, 1.864; $Mg(OH)_2$, 0.0165 g/100 g H_2O. These compositions almost agreed with those obtained on ternary system KCl-K_2SO_4-H_2O[16~18].

System KCl-$MgCl_2$-$Mg(OH)_2$-H_2O at 50° Though there has never been any report concerning this system too, concerning the ternary system excluding magnesium hydroxide from the system, some papers have been reported[12,19].

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13) M-L. Delyon, *Bl. Soc. Chim.*, [5], **3**, 1811 (1936).

14) A. Benrath, A. Schlömer, J. Clermont and S. Kojitsch, *Z. anorg. Chem.*, **170**, 269 (1884).

15) A. Benrath and H. Benrath, *ibid.*, **184**, 366 (1929).

16) E. Cornec and H. Hering, *Caliche*, **8**, 58 (1926).

17) W.C. Blasdale, *J. Ind. Eng. Chem.*, **10**, 344 (1918).

18) H. P. recht and B. Wittjen, *Ber.*, **14**, 1674 (1881).

19) J.D'Ans, "Die Lösungsgleichgewichte der Systeme der Salze ozeanischer Salzablagerungen" Kali-Forschungs-Anstalt G. m.b.H., Berlin, 1933, s. 81 und Tafel IV, fig. 2.

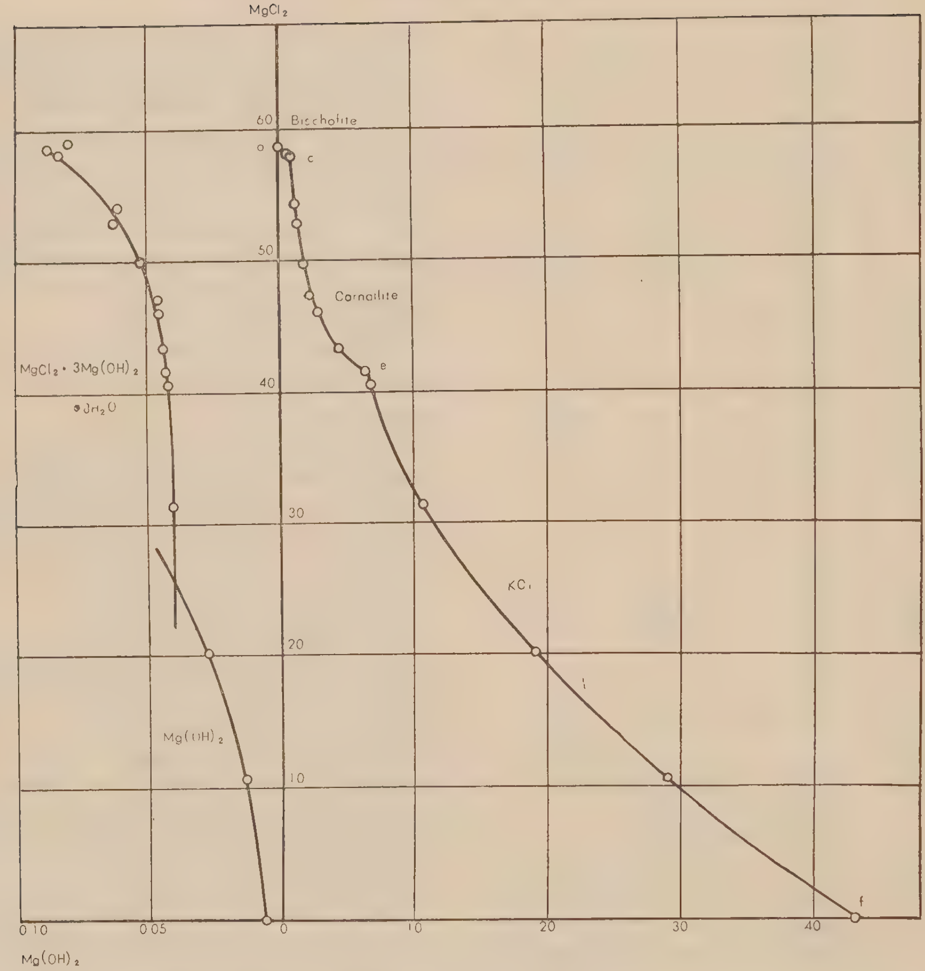

FIG. 6. The 50° Isotherm of the System KCl-$MgCl_2$-$Mg(OH)_2$-H_2O [g salts/100 g H_2O].

Results obtained from this system are represented in Fig. 6. The solubility of magnesium hydroxide was so small that the diagram must be divided into two parts, namely those of potassium chloride-magnesium chloride and those of magnesium chloride-magnesium hydroxide.

As shown in the left hand of Fig. 6, the solubility of magnesium hydroxide in this system, taking the concentration of magnesium chloride as the parameter, was closely analogous to those of the foregoing system $MgCl_2$-$Mg(OH)_2$-H_2O.

The solid phases of this system were bischofite, carnallite, basic magnesium chloride ($MgCl_2 \cdot 3\,Mg(OH)_2 \cdot 8\,H_2O$), potassium chloride and magnesium hydroxide.

The chemical compositions of liquid phases of monovariant points c and e, whose solid phases are (bischofite, carnallite and basic magnesium chloride) and (carnallite, potassium chloride and basic magnesium chloride) respectively, are

point c: KCl, 0.670; $MgCl_2$, 58.34; $Mg(OH)_2$, 0.0878 g/100 g H_2O
point e: KCl, 6.57 ; $MgCl_2$, 41.46; $Mg(OH)_2$,

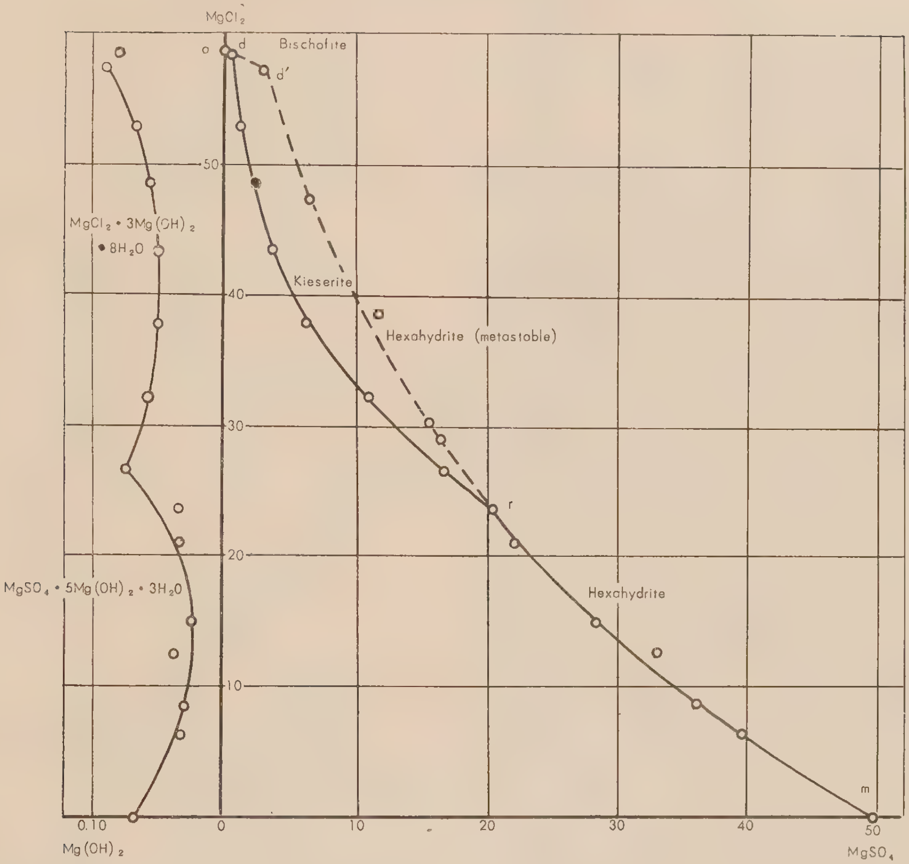

FIG. 7. The 50° Isotherm of the System $MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O. [g salts/100 g H_2O].

0.0426 g/100 g H_2O

These compositions excluding magnesium hydroxide are in close agreement with those described in other papers[12,19].

System $MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O at 50° Though there has never been any report concerning this system, concerning the ternary system excluding magnesium hydroxide from the system, polytherm is given by J.D′Ans[20].

Results obtained from this system are presented graphically in Fig. 7. The solubility of magnesium hydroxide being very small, the diagram must be divided into two parts, namely those of magnesium chloride-magnesium sulfate and those of magnesium chloride-magnesium hydroxide. The solid phases of the final stable state were bischofite, kieserite and hexahydrite, but at metastable state, kieserite did not appear. The solubility of kieserite is shown by the curve dr and the point r is the co-existent point of kieserite and hexahydrite. The solubility of hexahydrite at metastable state is shown by the curve d′r and the point d′ is the co-existent point of bischofite and hexahydrite. The time required to convert hexahydrite, the metastable solid phase, to kieserite, the stable solid phase, was about fifty days.

The chemical compositions of the liquid phase of monovariant points d, d′ and r are

point d: $MgCl_2$, 58.30; $MgSO_4$, 0.590; $Mg(OH)_2$, 0.147 g/100 g H_2O

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[20] J.D' Ans, *ibid.*, s. 128 und Tafel XI, fig. 1,2.

point d′: $MgCl_2$, 57.41; $MgSO_4$, 3.157; $Mg(OH)_2$, 0.0913 g/100 g H_2O
point r: $MgCl_2$, 23.71; $MgSO_4$, 20.48; $Mg(OH)_2$, 0.0366 g/100 g H_2O

These compositions of points d and r being plotted in polytherm given by J.D′Ans, there are in good agreement between these two data.

System K_2SO_4-$MgSO_4$-$Mg(OH)_2$-H_2O at 50° Though nothing has been reported concerning this system, concerning the ternary system excluding magnesium hydroxide from the system, one report was made by A. Benrath and H. Benrath[21].

The solid phases of this system being forecasted to NS salt B, basic magnesium sulfate, leonite, hexahydrite, potassium sulfate and magnesium hydroxide, the experiments have been carried out with caution of the following facts: (1) the width of the crystallization field of NS salt B in comparison with those of 100° isotherm; (2) chemical composition of basic magnesium sulfate is whether $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$, the same double salt deposited in isotherm of the foregoing system $MgSO_4$-$Mg(OH)_2$-H_2O and in 100° isotherm of this system in our study, or $MgSO_4 \cdot 3MgO \cdot 11H_2O$, reported by M-L. Delyon[13] being formed in 80° isotherm of the system $MgSO_4$-$Mg(OH)_2$-H_2O; (3) whether the unknown crystal deposited in 100° isotherm of this system is formed or not, etc..

Results obtained from this system are presented graphically in Fig. 8. The solubility of magnesium hydroxide being very small, the diagram must be divided into two parts, namely those of magnesium sulfate-potassium sulfate and magnesium sulfate-magnesium hydroxide.

The curved line bt was the border line of existence area of NS salt B and basic magnesium sulfate in stable equilibrium and the broken line b′t′ was the same line in metastable state. Therefore the crystallization area of NS salt B included metastable state was about twice as large as the stable area. The amount of potassium dissolved in liquid phase on the border line, however, was slightly higher than those of 100° isotherm and comparing the area of NS salt B in 100° isotherm with those in 50° isotherm, the former is somewhat larger than the latter.

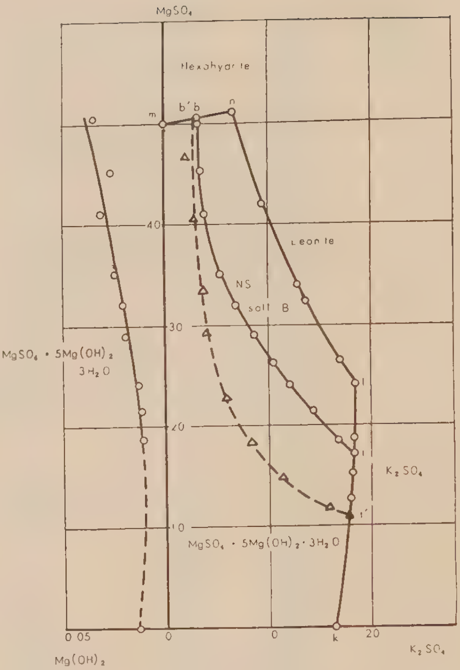

FIG. 8. The 50° Isotherm of the System K_2SO_4-$MgSO_4$-$Mg(OH)_2$-H_2O. [g salts/100 g H_2O].

The chemical composition of crystallized basic magnesium sulfate was determined to be $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$, the same double salt deposited in the foregoing system $MgSO_4$-$Mg(OH)_2$-H_2O, and $MgSO_4 \cdot 3MgO \cdot 11H_2O$ was not detected from anywhere in this phase diagram.

The unknown crystal deposited in 100° isotherm of this system was not formed from any solution included in this system.

The crystallization speed as well as the dissociation speed of basic double and triple salt in such solutions as included in the metastable area was extremely slow.

The solubility of magnesium hydroxide in

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[21] A. Benrath and H. Benrath, *Z. anorg. Chem.*, **189**, 79 (1930).

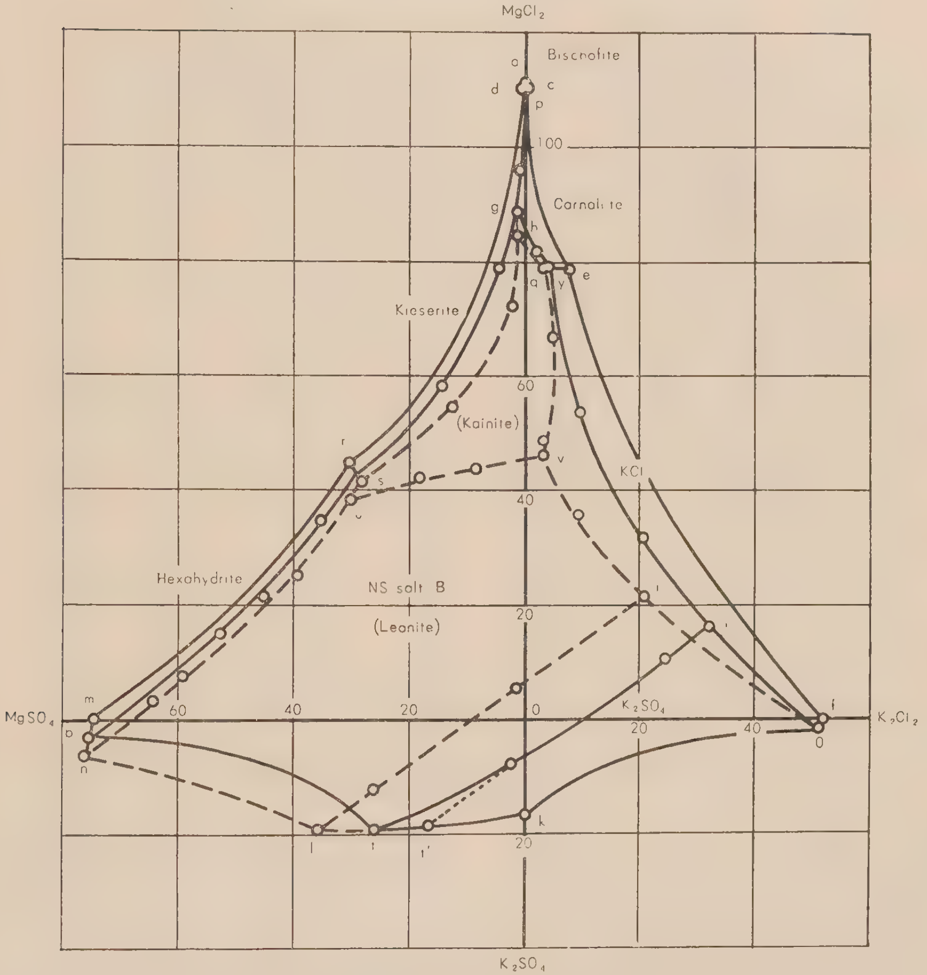

FIG. 9. The 50° Isotherm of the System KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O. [mol salts/1000 mol H_2O].

such solutions as their compositions are located on the border line bt in Fig. 8 increased gently with increasing dissolved magnesium sulfate.

The chemical compositions of liquid phases of monovariant points b, n, l, t and t′, whose solid phases are (hexahydrite, NS salt B and basic magnesium sulfate), (hexahydrite, leonite and NS salt B), (leonite, NS salt B and potassium sulfate), (potassium sulfate, NS salt B and basic magnesium sulfate) and (potassium sulfate, basic magnesium sulfate and NS salt B (metastable)) respectively, are

point b:	K_2SO_4, 3.491; $MgSO_4$, 50.51; $Mg(OH)_2$, 0.0343 g/100 g H_2O
point n:	K_2SO_4, 6.750; $MgSO_4$, 51.00; $Mg(OH)_2$, 0.0060 g/100 g H_2O
point l:	K_2SO_4, 18.61; $MgSO_4$, 24.05; $Mg(OH)_2$, 0.0174 g/100 g H_2O
point t:	K_2SO_4, 18.59; $MgSO_4$, 17.41; $Mg(OH)_2$, 0.0126 g/100 g H_2O
point t′:	K_2SO_4, 18.16; $MgSO_4$, 11.30; $Mg(OH)_2$, 0.0268 g/100 g H_2O

These compositions of points n and l being plotted in the isotherm reported by A. Benrath, and H. Benrath, there are good agreement be-

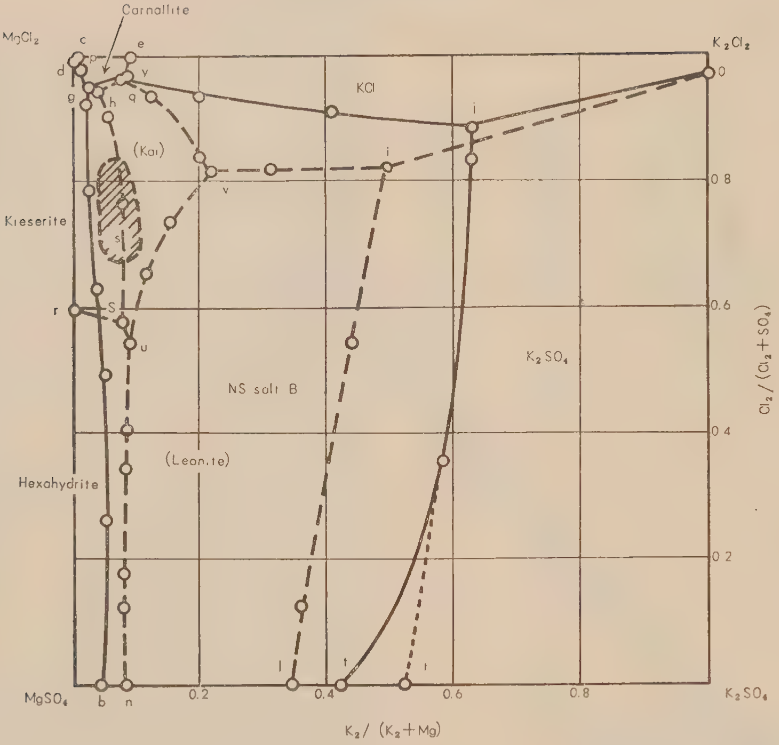

FIG. 10. The 50° Isotherm of the System KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O. [mol salts/1000 mol H_2O].

tween these two data.

System KCl-K_2SO_4-$MgCl_2$-$MgSO_4$-$Mg(OH)_2$-H_2O at 50° Heretofore though nothing has been reported concerning the isotherm of any temperature of this quinary system and also the 50° isotherm of quaternary system excluding magnesium hydroxide from this quinary system, concerning the 55° isotherm of the quaternary system one report was made by J.D'Ans and others[22]. Therefore the isotherm of quaternary system as well as the quinary system were studied.

Results obtained from both quinary and quaternary systems are shown in Table I and represented graphically in Figs. 9 and 10. As the amount of dissolved magnesium hydroxide was too small to be indicated, plotting was omitted in these figures. The broken lines in these figures represent the equilibrium of the quaternary system.

The crystallization field of NS salt B being very large as shown in Figs. 9 and 10, it should be possible to easily separate this salt from the solutions included in this system and moreover comparing the yield of potassium in the case of separation of NS salt B with in the case of leonite or kainite, the former should be greater than the latter ones because the saturation surface of the former is located below those of the latter ones.

The hatched area "S" in Fig. 10, represent the range of chemical compositions of various bitterns produced by salt manufacturing factories. The range "S" is wholly included in the crystallization field of NS salt B and partially in kainite field, therefore NS salt B could be directly separated from any bittern produced by salt manufacturing factories, while kainite could be separated only from some limited bitterns. Furthermore, potassium is included in kainite

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[22] J.D'Ans, A. Bertsch and A. Gessner, *Kali*, **9**, 195 (1915).

TABLE I. DATA FOR 50° ISOTHERM OF THE SYSTEM $KCl\text{-}K_2SO_4\text{-}MgCl_2\text{-}MgSO_4\text{-}Mg(OH)_2\text{-}H_2O$

Points of Figs. 9 and 10	Composition of Liquid Phase Mol per 1000 mol H_2O					Agitation Period	Solid Phase Remarks*
	K_2	Mg	Cl_2	SO_4	$(OH)_2$	Hrs	
	20.56	14.84	12.50	22.82	0.0815	670	Ks+NS.B
	33.04	19.44	43.69	8.764	0.0287	168	//
j	38.96	23.18	55.04	7.068	0.0364	168	Kc+Ks+NS.B
	26.08	37.61	57.92	5.738	0.0346	120	Kc+NS.B
	14.54	58.20	67.96	4.739	0.0395	120	//
	7.591	80.82	85.36	3.371	0.0625	168	Kc+Car.+NS.B
	4.712	84.58	86.36	2.720	0.2051	192	Car.+NS.B
g	2.392	92.78	90.99	4.132	0.0699	384	Kie.+Car.+NS.B
	2.012	85.86	80.70	6.640	0.1600	264	Kie.+NS.B+B.M.C.
	1.744	74.43	59.66	16.03	0.1695	240	//
	2.718	78.39	51.14	29.89	0.0101	192	Hex.+NS.B+B.M.C.
	4.096	74.20	38.81	39.39	0.0101	192	Hex.+NS.B.+B.M.S.
	3.837	71.39	18.49	56.68	0.0630	288	//
	18.70	32.57	6.351	44.87	0.0286	496	Ks+Leo.+NS.B
	22.58	29.40	27.65	24.35	0.0101	496	//
i	32.52	33.39	53.80	11.71	0.0122	288	Kc+Ks+Leo.+NS.B
	22.04	48.41	57.90	12.54	0.0062	288	Kc+Leo.+NS.B
v	17.00	60.25	63.01	14.02	**	360	Kc+Leo.+Kai.
	15.86	61.35	64.26	12.85	0.0048	312	Kc+Kai.+NS.B
	10.51	72.08	76.78	5.554	0.0127	312	//
g	7.014	82.45	85.65	3.506	0.0307	360	Kc+Car.+Kai.+NS.B
h	3.671	90.05	88.23	5.303	**	336	Kie.+Car.+Kai.
	3.241	90.30	76.97	4.988	**	240	Kie.+Car.
	4.997	79.69	60.82	7.718	**	384	Kie.+Kai.
	6.417	73.68	48.25	18.98	0.0095	408	//
s	6.636	76.92	31.72	35.14	0.0048	192	Kie.+Hex.+Kai.+NS.B
	6.776	71.39	27.68	46.30	0.0111	312	Hex.+Leo.+NS.B
	6.466	73.00	9.874	51.68	0.0074	168	//
	6.226	73.35	14.14	65.70	0.0090	192	//
	6.533	74.42	9.874	71.01	0.0122	168	//
	11.79	63.97	55.41	20.25	**	336	Leo.+Kai.
	8.933	69.47	51.24	27.20	**	240	//
u	6.892	75.42	45.23	37.30	**	432	Hex.+Leo.+Kai.
	1.166	98.25	97.67	1.803	**	264	Kie.+Car.
p	0.458	111.28	110.74	0.996	**	360	Kie.+Bis.+Car.

* Chemical formulae and names of solid phases are abbreviated as follows; Kc=KCl, Ks=K_2SO_4, Bis.=Bischofite, Car.=Carnallite, Hex.=Hexahydrite, Kai.=Kainite, Kie.=Kieserite, Leo.=Leonite, B.M.C.=Basic Magnesium Chloride $MgCl_2 \cdot 3Mg(OH)_2 \cdot 8H_2O$, B.M.S.=Basic Magnesium Sulfate $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$, NS.B=NS salt B $K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$

** As this point belongs to the quaternary system $KCl\text{-}K_2SO_4\text{-}MgCl_2\text{-}MgSO_4\text{-}H_2O$, hydroxide is absent.

as chloride while in NS salt B as sulfate.

From these experimental data, in order to separate potassium dissolved in bittern as sulfate directly at 50°, it should be concluded that the only one method is to separate it as NS salt B. Calculating the yield of NS salt B from bittern at 50° by these phase diagrams, about seventy eight per cent of dissolved potassium is separated as NS salt B. Though this calculated yield of potassium is relatively large compared with those of 100° isotherm (yield is calculated about fifty per cent), crystallization speed of NS salt B at 50° being very small, its separation at 50° might be somewhat doubtful economically.

Acknowledgements. The author is grateful to Prof. Dr. Jiro Sugi and Prof. Dr. Shumpei Oka of Tokyo University for their kind advice and encouragement throughout the course of this study. Indebts are also due to Mr. T. Matsuo and Miss S. Takabayashi for their assistance in carrying out this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 372~381, 1960]

Studies on the Metabolic Products of *Rosellinia necatrix* Berlese

Part I. Isolation and Characterization of Several Physiologically Active Neutral Substances

By Yu-Shih Chen

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received September 30, 1959

Three kinds of diketopiperazines which have retarditive activity for the growth of plant seedlings and plant roots at concentrations ranging from 1 : 2,500 to 1 : 100,000, were isolated from the neutral fraction by extracting the cultured broth of *Rosellinia necatrix*. These three diketopiperazines have been proved to be L-prolyl-L-leucine anhydride, L-prolyl-L-valine anhydride and L-prolyl-L-phenylalanine anhydride respectively, and the last one seems to be a new diketopiperazine.

Furthermore, a crystalline wax having m.p. 52°C, a physiologically inactive substance, was also isolated from the same neutral fraction and presumed to be the saturated hydrocarbon of *n*-pentacosane $C_{25}H_{52}$.

It is well known that many kinds of phytopathogene produce toxic metabolites. Tamari and Kaji[1] isolated α-picolic acid and piricularin from the cultured broth of *Piricularia oryzae* Cav., which causes blast disease of rice. They also proved that the toxic metabolites are produced in the rice plant infected by blast.

Recently, the present author[2] reported that *p*-hydroxy-phenylacetic acid is the main toxic metabolite of *Corticium sasakii*, the causal fungus of sheath blight of rice plant.

White root rot (Shiromonpa-byô) incited by *Rosellinia necatrix* Berlese is widely distributed in Japan. In this country tea plants, ramie and orchard trees such as apple, pear, mulberry, persimmon, chestnut, grape, peach, plum, loquat and orange trees, are seriously affected by this disease. The fungus infects the roots forming a superficial, whitish web of mycelium on the surface. It destroys the roots and causes the tree to be gradually weakened, accompanied with yellowing and premature fall of the leaves, and to be finally killed.

This work was originated from an assumption that the pathological effect on the plant may be involved with certain metabolic products of the pathogene, and the author has attempted to isolate the physiologically active substance.

Six crystalline compounds, namely compounds A, B, C, D, E and a new acidic substance, were isolated from the extracts of the cultured broth of *Rosellinia necatrix*.

The pathogene, *Rosellinia necatrix*, was cultured for 40~50 days at about 20°C on the glucose-pepton medium containing thiamine and biotin.

The filtrated broth was first neutralized and then extracted with chloroform. The concentrate of the chloroform extract was then applied on an alminium oxide column and developed with petroleum ether, ethylacetate-benzene (2:8, v/v), ethylacetate-benzene (3:8, v/v) and ethylacetate-benzene (3:7, v/v) in the order described.

Compound A, (m.p. 52°C), colorless scaly wax, which was obtained from the petroleum ether

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[1] K. Tamari and J. Kaji, *J. Agr. Chem. Soc. Japan*, **28**, 254~258 (1954).

[2] Yu-Shih Chen, This Bulletin, **22**, 136~142 (1958).

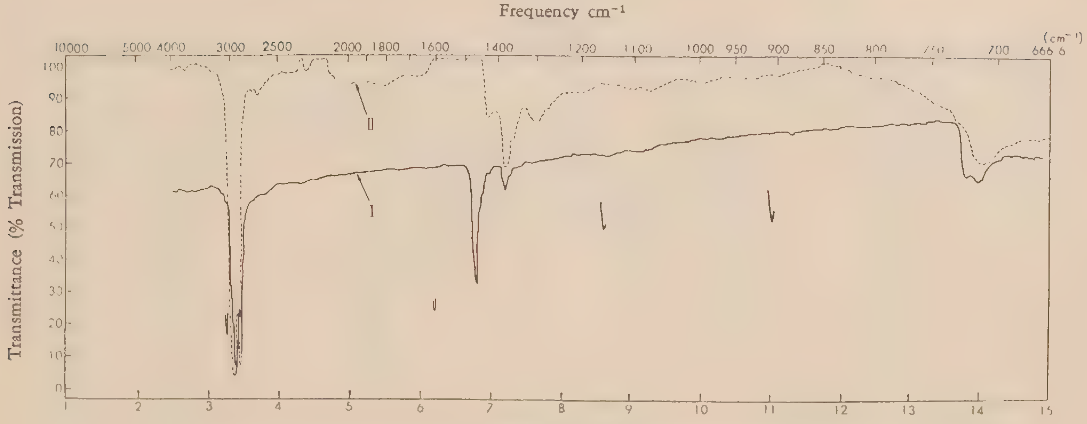

FIG. 1. Infrared Spectra of Conpound A (n-Pentacosane $C_{25}H_{52}$).
I. Spectrum as Nujol-mull. II. Spectrum in Carbon disulfide.

eluate, was found to be a saturated hydrocarbon.

From the ethylacetate-benzene (2:8) eluate, the second neutral compound B, (m.p. 168~170°C), was obtained in colorless plates.

Ethylacetate-benzene (3:8) eluate was found to contain two substances and was further purified on silica-gel column. Compound C, (m.p. 158~161°C), was obtained as colorless plates. Afterwards, compound D, (m.p. 191~193°C), was eluted from the column with chloroform in colorless needles.

From the last eluate of the alminium oxide column with ethylacetate-benzene (3:7), compound E, (m.p. 127~128°C), was also obtained as colorless needles.

In the process of isolation and purification by chromatography, the separated compounds were assayed biologically using plant seedlings to check the physiological activity. Every compound except compound A was found to retard the growth of roots and leaves of rice at concentrations ranging from 1:2,500 to 1:100,000.

Furthermore, an additional unknown acidic compound (m.p. 206~208°C), was obtained as white needles by ether extraction of the acidified aqueous layer after the above coloroform extraction. This acidic compound also showed strong retarditive activity. The author wishes to propose the name rosellinic acid for this compound.

Compound A is a neutral colorless scaly wax, m.p. 52°C, soluble in benzene, chloroform, n-hexane, ligroin, petroleum ether and hot acetone, but insoluble in water, alcohols and ethylacetate. It is very stable in either concentrated acid or alkali, and gave negative results with all the color reactions tested. Elementary analysis and molecular weight determination indicated that it is a compound of $C_{25}H_{52}$. It melts in the same range of n-pentacosane and showed identical infrared spectrum with that of n-pentacosane (characteristic band of more than six successive methylene groups at about 730~710 cm^{-1}) (Fig. 1). Consequently, compound A is considered to be n-pentacosane. Although the compound is none toxic to the plant, it is interesting to find that *Rosellinia necatrix*, a phytopathogene, produces such a saturated hydrocarbon.

Chibnall[3,4] has shown that the higher numbers of wax paraffins, with 25~35 carbon atoms of an odd number, are the common constituents of plant and insect waxes. These constituents probably arise through the decarboxylation of higher fatty acids, since all natural acids have

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3) A.C. Chibnall, S.H. Riper, A, Pollard, E.F. Williams and P.N. Sahai, *Biochem. J.*, **28**, 2189~2208 (1934).

4) Fieser & Fieser, Organic Chemistry, p. 36 (1952).

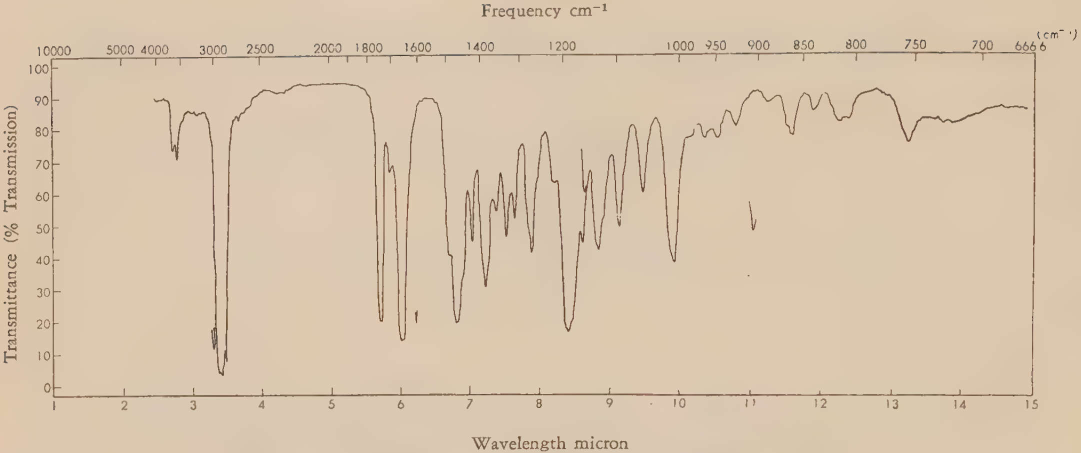

FIG. 2. Infrared Spectrum of Compound B as Nujol-mull.

an even number of carbon atoms. Therefore, *n*-pentacosane $C_{25}H_{52}$ probably arises through decarboxylation of cerotic acid $C_{25}H_{51}COOH$ which is a characteristic component of many waxes.

Compound B, plates, is a neutral substance containing nitrogen, melts at 168~170°C, and is easily soluble in alcohols, chloroform, acetone, benzene and ethylacetate, and sparingly soluble in ether, but insoluble in water, ligroin and petroleum ether. It reacts with 2,4-dinitrophenylhydrazine, tetranitromethane and dilute potassium permanganate, but gives a negative ninhydrin reaction.

The ultraviolet spectrum of compound B in methanol showed absorption peak (shoulder) at near 280 mμ (ε 87). The infrared spectrum as Nujol mull showed the characteristic band of free hydroxyl group at 3600 and 3540 cm^{-1} (resistant to acetylation), that of five membered ring ketone at 1753 cm^{-1}, that of amide linkage at 1667 and 1656 cm^{-1}, and in hexachloro 1,3-butadiene exhibited the band of 2968 and 1375 cm^{-1} (Fig. 2).

On hydrolysis with concentrated mineral acid, it yielded an amino acid ($C_8H_{17}O_2N$), which reacted with ninhydrin.

Further study of compound B is in active progress, and the result will be reported latter.

Compounds C, D and E are all closely related to diketopiperazines. They react with picric acid and *m*-nitrobenzene described by Abderhalden and Komm⁵⁾, and also with potassium mercuric iodide⁶⁾, but give a negative ninhydrin reaction before hydrolysis.

Compound C is optically active, $[\alpha]_D^{20} = -141°$ (c, 3.33 in ethanol), and has a molecular formula, $C_{11}H_{18}O_2N_2$. It is easily soluble in alcohols, acetone, chloroform and hot water, and sparingly soluble in cold benzene, ether and ethylacetate, but insoluble in ligroin and petroleum ether.

The ultraviolet spectrum of compound C in methanol showed no significant absorption peak in the wavelength range of 221~400 mμ, but exhibited a rather strong end absorption. The infrared spectrum as Nujol mull exhibited two strong bands at 1670 and 1635 cm^{-1} (amide CO), a single band at 3260 cm^{-1} (bonded NH), the absence of the characteristic band of the peptide likage at about 1550 cm^{-1} and the lack of strong C–O absorption in the lower frequency region (1250~1000 cm^{-1}) (Fig. 3).

Evidence described above indicated a cyclic monosubstituted or a disubstituted amide structure.

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5) E. Abderhalden and E.Z. Kom, *Physiol. Chem.*, **139**, 181 (1924).

6) E. Abderhalden and R.Z. Haaf, *ibid.*, **151**, 114 (1926).

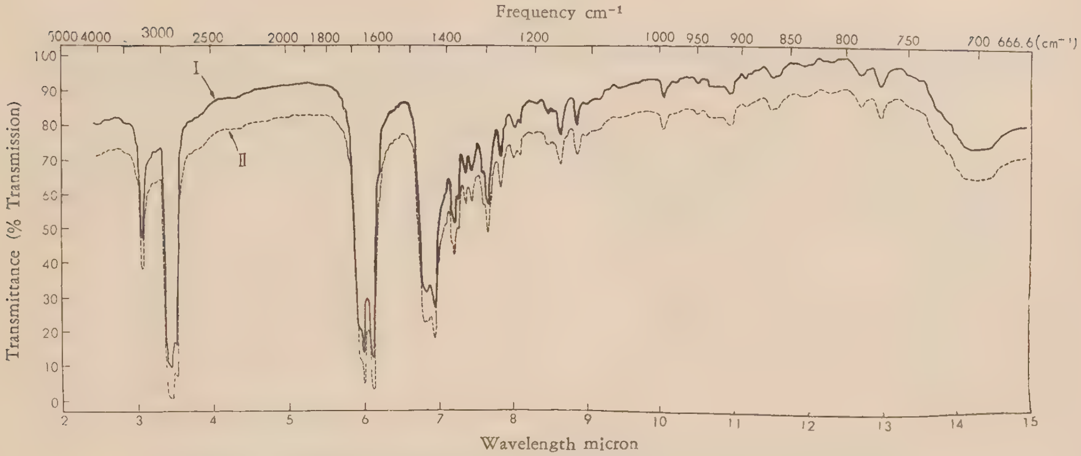

FIG. 3. Infrared Spectra of Compound C (L-Prolyl-L-Leucine Anhydride).

I. Isolated compound from fungus. II. Synthetic compound.

On hydrolysis with conc. HCl, it gave only two amino acids which were identified to be leucine and proline by paper chromatography, and both of these amine acids were proved to be in the L-form by the use of D-amino acid oxidase.

Identification of compound C as L-prolyl-L-leucine anhydride (diketopiperazine) was further confirmed by mixed melting point test and accordance of infrared spectra with a synthetic sample.

In the literature it is reported that this material has first isolated been from hog adrenal cortex in 1945[7], and since then has been isolated from culture filtrates of a streptomycin producing strain of *Streptomyces* and *Aspergillus fumigutus*[8].

Compound D with molecular formula $C_{10}H_{16}O_2N_2$ was also found to be optical active: $[\alpha]^{20}_{D} = -161°$ (c, 1 in ethanol). It is a neutral colorless needle (from ethylacetate-ligroin), m.p. 191～193°C, and soluble in acetone, alcohols, benzene, chloroform, ethylacetate and cold water, very slightly soluble in ether, but insoluble in *n*-hexane, ligroin and petroleum ether.

It showed a similar ultraviolet spectrum to that of compound C.

The infrared spectrum as Nujol mull exhibited a strong band at 1660 cm^{-1} (amide CO), a single band at 3260 cm^{-1} (bonded NH), with the absence of the characteristic band of the peptide linkage at about 1550 cm^{-1} and a lack of strong C–O absorption in the lower frequency region (1250～1000 cm^{-1}) (Fig. 4). This indicated a cyclic monosubstituted or a disubstituted amide structure.

Paper chromatography of the acid hydrolysate confirmed the identity of only two amino acids in compound D to be valine and proline, and both of these amino acids were proved to be the L-form by the use of D-amino acid oxidase.

The identification of the compound D with L-prolyl-L-valine anhydride was further established by mixed melting point test and in accordance with infrared spectra with a synthetic sample.

Koaze[9] described the isolation of L-prolyl-L-valine anhydride from the culture filtrate of *Streptomyces* sp. S–580, and showed that germination of the rice seed could be promoted by this compound at concentrations ranging from 1 : 2,500 to 1 : 100,000.

An optical active compound E, $[\alpha]^{20}_{D} = -99.8°C$ (c, 1 in ethanol), with a molecular formula of

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[7] M.H. Kuizenga, J.W. Nelson, S.C. Lyster and D.J. Ingle, *J. Biol. Chem.*, **160**, 15～24 (1945).

[8] J. L. Johnson, W. G. Jackson and T.E. Elbe, *J.A.C.S.*, **73**, 2947～2948 (1951).

[9] Y. Koaze, This Bulletin, **22**, 98～103 (1958).

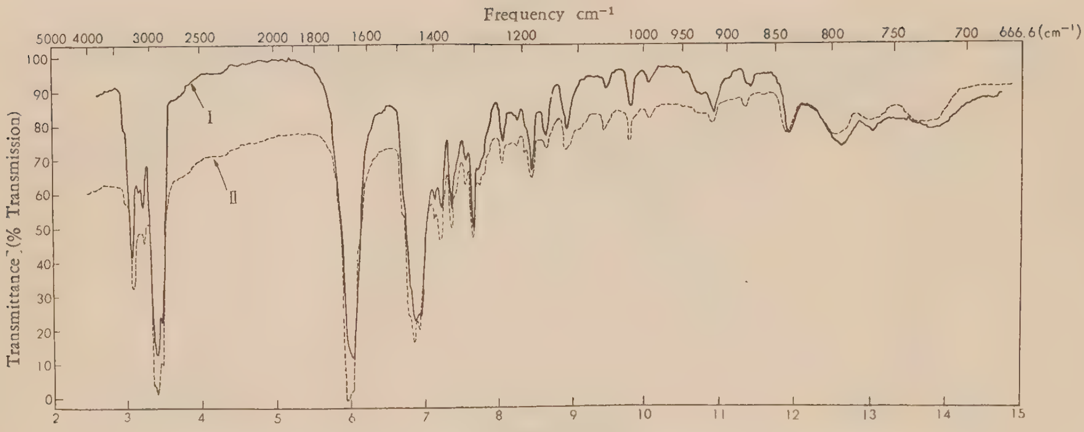


FIG. 4. Infrared Spectra of Compound D (L–Prolyl–L–Valine Anhydride).
I. Isolated compound from fungus. II. Synthetic compound.

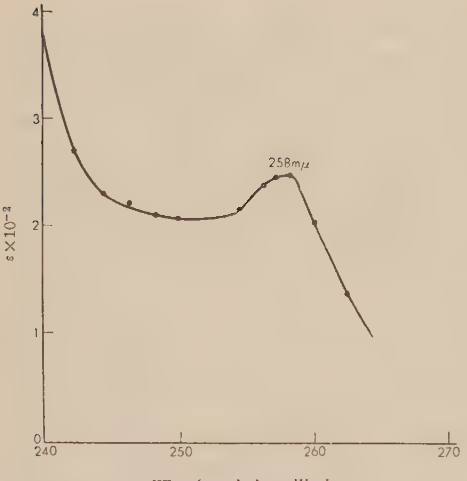


FIG. 5. U.V. Spectrum of L–Prolyl–L–Phenylalanine Anhydride.

$C_{14}H_{16}O_2N_2$ was obtained as neutral colorless needles (from ethylacetate-ligroin), m.p. 127～128°C. It is soluble in alcohols, benzene, chloroform, ethylacetate, acetone and cold water, and very slightly soluble in ether, but insoluble in ligroin and petroleum ether.

The ultraviolet spectrum (Fig. 5) in methanol exhibited an absorption maximum at wavelength 258 mμ (ε 244).

The infrared spectrum as Nujol mull showed a characteristic band of bonded NH at 3300, that of C–O at 1705 and 1690, that of amide C–O at 1660 and 1640, that of the aromatic ring at 1610 and 1505, that of aromatic monosubstitute at 760 and 700 cm⁻¹, the absence of characteristic band of the peptide linkage at about 1550 cm⁻¹, and a lack of strong C–O absorption in the lower frequency region (1250～1000 cm⁻¹) (Fig. 6). This indicated a cyclic disubstituted amide structure containing the aromatic ring in the molecule.

The acid hydrolysate gave only two amino acids, phenylalanine and proline, determined by paper chromatography, and both of these amino acids were proved to be the L-form by the use of D-amino acid oxidase.

Compound E was further identified to be L-prolyl-L-phenylalanine anhydride by mixed melting point test and accordance of infrared spectra with a synthetic sample.

According the author's knowledge, L-prolyl-L-phenylalanine anhydride has never been reported to be found in nature. Although it is closely related to compounds C and D, it is a new derivative of diketopiperazine.

An attempt made to isolate compounds A, B, C, D, E and the acidic compound described above from uninoculated culture medium resulted in failure.

Therefore, compounds A, B, C, D, E and rosellinic acid appear to be the true metabolite.

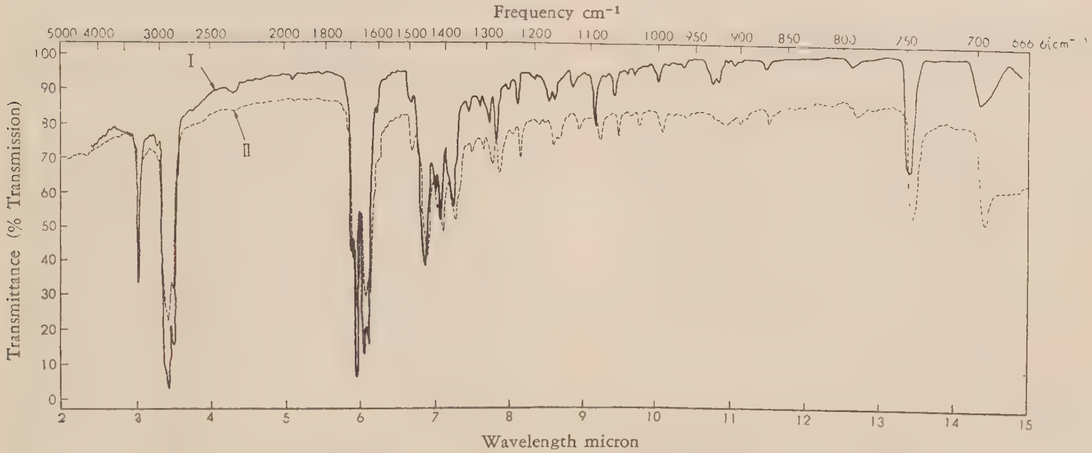
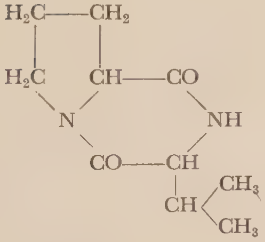


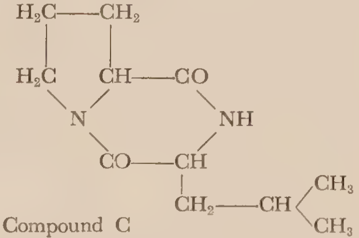
FIG. 6. Infrared Spectra of Compound E (L-Proly-L-Phenylalanine Anhydride).
I. Isolated compound from fungus. II. Synthetic compound.

$CH_3(CH_2)_{23}CH_3$

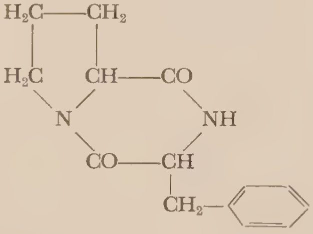
Compound A
m.p. 52°C
n-pentacosane



Compound D
m.p. 191~193°C
L-prolyl-L-valine anhydride



Compound C
m.p. 158~161°C
L-prolyl-L-leucine anhydride



Compound E
m.p. 127~128°C
L-prolyl-L-phenylalanine anhydride

EXPERIMENTAL*

1. Isolation of the toxic substances from the cultured broth.

The culture of *Rosellinia necatrix* used in this work was furnished by Mr. K. Kasai, Tea division of National Tokai-Kinki Agr. Expt. Station.

For isolation of metabolite, it was grown at 20~22°C for about 40~50 days on glucose-pepton medium containing thiamine and biotin.

Composition of the culture medium is shown in the following Table I.

Each 1.2 l-aliquot of the medium was poured onto a 2-l round flask. Bioassay was performed using young seedlings of rice or mung bean as the test plant. The seedlings similar in height were transferred to a Petri dish covered with 1% agar containing the filtrate of the cultured broth and an aliquot from each fraction in the process of isolation, to observe whether inhibiting effect will appear.

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* All values of melting points are uncorrected.

TABLE I. THE COMPOSITION OF THE CULTURE MEDIUM

Glucose	40 g
Pepton	5 g
KNO_3	10 g
K_2HPO_4	5 g
$MgSO_4 \cdot 7H_2O$	2.5 g
$FeSO_4$	50 mg
$ZnSO_4$	50 mg
Thiamine	20 mg
Biotin	200 γ
Tap water	1 l

Adjusted to pH 5.5 by aqueous NaOH.

From this filtrate of the cultured broth, five neutral substances of compounds A, B, C, D and E, and a new acidic substance, namely rosellinic acid with m.p. 206~208°C were obtained. Details of this procedure is shown in Fig. 7. All of these substances except compound A acted as the growth inhibiting factor at concentrations ranging from 1:2,500 to 1:100,000.

The yield from 10 l filtrate was 80 mg of compound A, 13 mg of compound B, 25 mg of compound C, 40 mg of compound D, 60 mg of compound E and 120 mg of rosellinic acid, as pure form.

2. Elementary analysis and molecular weight determination of compound A.

Analytical data of compound A:

Anal. Found: C 84.89, H 14.36

Calcd. for $C_{25}H_{52}$: C 85.14, H 14.86

The molecular weight determined by the micro-Rast method was found to be 342 (calcd. 352).

3. Elementary analysis, molecular weight determination and hydrolysis of compound B.

Analytical data of compound B:

Anal. Found: C 62.68, H 8.81, N 5.48

Calcd. for $C_{14}H_{21}O_4N$: C 62.90, H 7.92, N 5.24

Calcd. for $C_{14}H_{23}O_4N$: C 62.43, H 8.61, N 5.20

Calcd. for $C_{14}H_{25}O_4N$: C 61.96, H 9.29, N 5.16

The molecular weight determined by the micro-Rast method was found to be 253 (calcd. 267~271).

Compound B (80 mg) was hydrolyzed with about fourty fold (v/w) 36% HCl at 120±2°C for twenty hours in a sealed glass tube. The dark colored mixture was

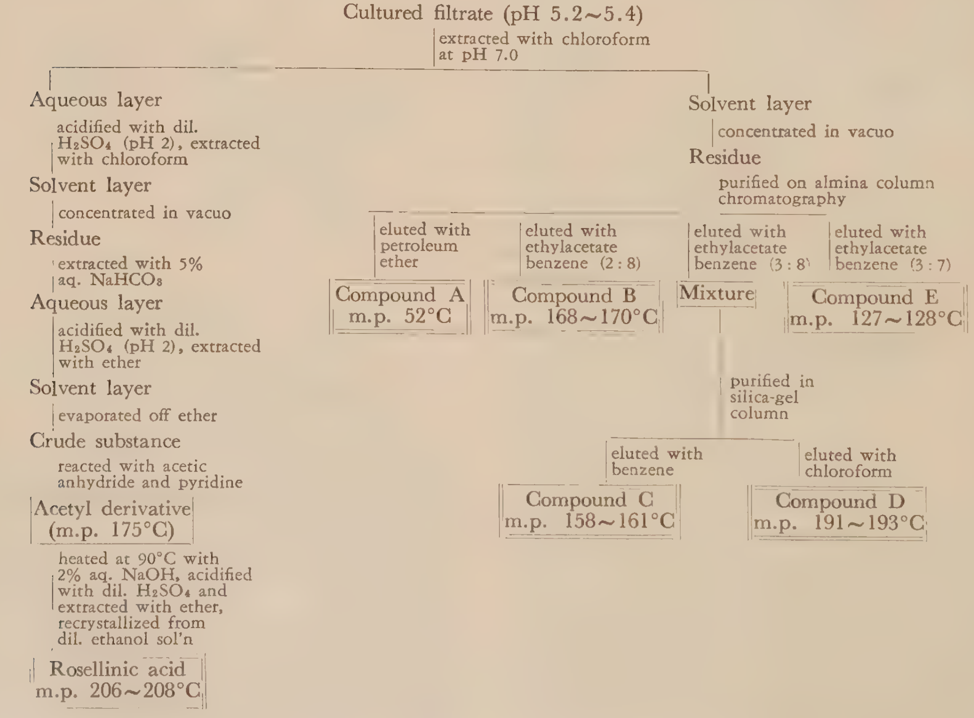
Cultured filtrate (pH 5.2~5.4)
extracted with chloroform at pH 7.0
Aqueous layer
acidified with dil. H_2SO_4 (pH 2), extracted with chloroform
Solvent layer
concentrated in vacuo
Residue
extracted with 5% aq. $NaHCO_3$
Aqueous layer
acidified with dil. H_2SO_4 (pH 2), extracted with ether
Solvent layer
evaporated off ether
Crude substance
reacted with acetic anhydride and pyridine
Acetyl derivative (m.p. 175°C)
heated at 90°C with 2% aq. NaOH, acidified with dil. H_2SO_4 and extracted with ether, recrystallized from dil. ethanol sol'n
Rosellinic acid m.p. 206~208°C
Solvent layer
concentrated in vacuo
Residue
purified on almina column chromatography
eluted with petroleum ether
eluted with ethylacetate benzene (2:8)
eluted with ethylacetate benzene (3:8)
eluted with ethylacetate benzene (3:7)
Compound A m.p. 52°C
Compound B m.p. 168~170°C
Mixture
Compound E m.p. 127~128°C
purified in silica-gel column
eluted with benzene
eluted with chloroform
Compound C m.p. 158~161°C
Compound D m.p. 191~193°C

Fig. 7. The Separation Procedure of the Metabolites of *Rosellinia necatrix*

evaporated to dryness after being treated three times with about 3 cc of distilled water. The residue was taken up in water (about 5 cc) and the insoluble humin-like material was separated.

The soluble fraction was passed through the anion exchange resin (Amberite IR 4B) in order to remove hydrogen chloride and was evaporated to dryness. The colorless residue (45 mg) was applied to the cellulose column by partition chromatography for isolation of the constituents of compound B.

The chromatographic column (14 × 280 mm) was placed with cellulose powder by the wetting method, which previously had been successively washed with 1 N HCl, water and then the upper layer of butanol : acetic acid : water (4 : 1 : 5, v/v). The hydrolysate, dissolved in 1.5 cc of the solvent mixture, was placed on the column and the column was developed with the upper layer of the above solvent mixture at a rate of 2.4 cc/hour. Each 0.6 cc eluate was serially collected by means of an automatic fraction collector and reacted with ninhydrin reagent separately.

Fractions 17∼35 gave positive reaction. The α-amino acid crystallized out from the combined eluate melted at 178°C.

Analytical data of amino acid after hydrolysis:

(a) Free acid.

Anal. Found: C 60.25, H 10.84, N 9.01
Calcd. for $C_8H_{17}O_2N$: C 60.34, H 10.76, N 8.80

(b) Acid hydrochloride.

Anal. Found: C 48.79, H 9.28, N 7.45, Cl 17.82
Calcd. for $C_8H_{17}O_2NHCl$: C 49.15, H 9.20, N 7.16, Cl 18.10

4. Elementary analysis, molecular weight determination and hydrolysis of compound C.

Analytical data of compound C:

Anal. Found: C 62.87, H 8.49, N 13.25
Calcd. for $C_{11}H_{18}O_2N_2$: C 62.81, H 8.63, N 13.33

The molecular weight determined by the micro-Rast method was found to be 238 (calcd. 210).

Compound C was hydrolyzed with about fourty fold (volume/weight) 36% HCl at 120±2°C for twenty hours in a sealed glass tube. The hydrolysate was subjected to repeating evaporation with water, over solid NaOH in vacuo, to remove excess HCl for further study.

(a) Detection of amino acids:

The paper chromatographic method (filter paper, Toyoroshi-No. 51: developing solvent, upper layer of *n*-butanol: acetic acid: water = 4 : 1 : 5 v/v, or isoamyl alc.: pyridine: water = 35 : 35 : 30 v/v) was applied.

The development was carried out by the ascending method for twenty to twenty-four hours at room temperature, and the developed strip was sprayed with ninhydrin (0.1% ninhydrin in *n*-butanol saturated with water).

As a result, the hydrolysate gave only two amino acids of leucine and proline.

(b) Determination of the configuration of these two amino acids:

Activity of D-amino acid oxidase towards the hydrolysate, determined by the manometric method, was negative. Therefore, both of these amino acids have the L-configuration.

(c) Quantitative determination of these two amino acids:

The photometric method[10], at the wavelength of 570 mμ for L-leucine and at 430 mμ for L-proline, was applied. The ratio of L-leucine to L-proline was found to be 1 mole to 1 mole in the hydrolysate mixture.

5. Elementary analysis, molecular weight determination and hydrolysate of compound D.

Analytical data of compound D:

Anal. Found: C 61.06, H 8.12, N 14.03
Calcd. for $C_{10}H_{16}O_2N_2$: C 61.20, H 8.22, N 14.28

The molecular weight determined by the micro-Rast method was found to be 212 (calcd. 196).

Compound D was hydrolyzed with about fourty fold (v/w) 36% HCl at 120±2°C for twenty hours in a sealed glass tube. The hydrolysate was treated as mentioned above for the following study.

(a) Detection of amino acids.

By paper chromatography, valine and proline were found to be the only two amino acids present in the hydrolysate.

(b) Determination of the configuration of these two amino acids.

Activity of D-amino acid oxidase towards the hydrolysate, determined by the manometric method, was negative. Therefore, both of these two amino acids have the L-configuration.

(c) Quantitative determination of these two amino acids.

By application of the same technique mentioned above, the ratio of L-valine to L-proline was found to be one to one.

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[10] S. Moore and W.H. Stein, *J.B.C.*, **211**, 907∼909 (1954).

6. Elementary analysis, molecular weight determination and hydrolysis of compound E.

Analytical data of compound E:

Anal. Found: C 68.90, H 6.41, N 11.68

Calcd. for $C_{14}H_{16}O_2N_2$: C 68.80, H 6.60, N 11.50

The molecular weight determined by the micro-Rast method was found to be 236 (calcd. 244).

Compound E was hydrolyzed with about fourty fold (v/w) 36% HCl at 120±2°C for twenty hours in a sealed glass tube. The hydrolysate was treated for the following study as mentioned above.

(a) Detection of amino acids:

By paper chromatography, phenylalanine and proline were found to be the only two amino acids present in the hydrolysate.

(b) Determination of the configuration of these two amino acids:

Activity of D-amino acid oxidase towards the hydrolysate, determined by the manometric method, was negative. Consequently, both of these two amino acids have the L-configuration.

(c) Quantitative determination of these two amino acids:

By the same technique mentioned above, the ratio of L-phenylalanine to L-proline was found to be 1 mole to 1 mole.

7. Synthesis of L-prolyl-L-valine anhydride (compound D)[7,11].

L-Valine (1 g) was dissolved in 20 cc of absolute methanol and treated with dry HCl gas for an hour. After removing the alcohol, the substance was recrystallized from alcohol-ether, and gave long needles of L-valine methylester-hydrochloride[12].

L-Proline (1 g) was dissolved in 10 cc of 2 N NaOH, and the solution was treated in an ice-bath, first with 1.5 g of carbobenzoxychloride and then with an additional 10 cc of 1 N NaOH in two portions with shaking. After twenty minutes the mixture was acidified to congo red, and a syrupy carbobenzoxy-L-proline was obtained. The carbobenzoxy compound was dissolved in 5 cc dry ether and 1.5 g PCl_5 was added to this solution at 0°C. After shaking gently for 15 min., most of the PCl_5 had dissolved. The etherial solution was decanted from undissolved PCl_5 and washed twice, quickly, with ice water and partially dried.

To this solution was added an ethylacetate solution of L-valine methylester, liberated from its hydrochloride by aqueous K_2CO_3.

Saturated aqueous $KHCO_3$ was added and the mixture was shaken intermittently for the next 20 minutes. After standing for further 40 min., water was added and the organic layer washed successively with 1 N HCl and 7% (w/v) aqueous $KHCO_3$. After drying and evaporation of the solvent, the syrupy carbobenzoxy-L-prolyl-L-valine methylester resulted.

The whole carbobenzoxy compound was dissolved in a mixture of 1 cc glacial acetic acid, 10 cc water, and was then hydrogenated at atmospheric pressure and room temperature in the presence of 0.1 g active Pd. black. The solvent mixture was evaporated to dryness under reduced pressure and the residue dissolved in dry methanol was saturated with ammonia under cooling. After one hour, methanol was evaporated off and the product recrystallized from ethylacetate-ligroin as fine needles: yield 0.24g (15.6%), m.p. 191~193°C, $[\alpha]_D^{20}=-160.5°$ (c, 1 in ethanol)

Anal. Found: C 61.24, H 8.18, N 14.16

Calcd. for $C_{10}H_{16}O_2N_2$: C 61.20, H 8.22, N 14.28

8. Synthesis of L-prolyl-L-leucine anhydride (compound C).

L-Leucine methylester hydrochloride was prepared in a nearly theoretical yield from L-leucine (1 g) by methanol and dry HCl gas.

The syrupy carbobenzoxy-L-proline was treated with PCl_5, and coupled with L-leucine methylester in a manner similar to that described above.

The syrupy carbobenzoxy-L-prolyl-L-leucine methylester was first hydrogenated and then subjected to dehydration with gaseous ammonia in methanol solution to effect the ring closure, and the reaction product recrystallized from acetone-ether as colorless plates: yield 0.35 g (22.6%), m.p. 160~161°C, $[\alpha]_D^{20}=-141°$ (c, 3.33 in ethanol).

Anal. Found: C 62.78, H 8.52, N 13.42

Calcd. for $C_{11}H_{18}O_2N_2$: C 62.81, H 8.63, N 13.42

9. Synthesis of L-prolyl-L-phenylalanine anhydride (compound E)[13].

L-Phenylalanine (1 g) was dissolved in 20 cc of absolute methanol and treated with dry HCl gas for one hour. After removing the alcohol, L-phenylalanine methylester hydrochloride was obtained as syrup.

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[11] R.L.M. Synge, *Biochem. J.*, **42**, 99~104 (1948).

[12] F. Fischer, *Ber.*, **34**, 433~454 (1901).

[13] J. I. Harris and T.S. Work, *Biochem. J.*, **46**, 196~199 (1950).

The methylester hydrochloride in dry ether was cooled to −10°C and an etherial solution of carbobenzoxychloride was added dropwise with stirring during the next 45 minutes. The reaction mixture was left overnight at room temperature and the reacted L-phenylalanine methylester hydrochloride separated by filtration.

The carbobenzoxy-L-phenylalanine methylester was obtained as an oil after removal of the solvent from the filtrate. This oily material suspend in methanol was then treated with an excess of 50% hydrazine hydrate and left for 24 hours at room temperature. The carbobenzoxy-L-phenylalanine hydrazide thus precipitated on addition of water was collected by filtration. The hydrazide in a mixture of 10 cc of glacial acetic acid and 20 cc of 2 N HCl was cooled to 0°C and to this aqueous solution of $NaNO_2$ (80 mg) was added dropwise in the next 10 min. under stirring.

The azide precipitates were collected and dissolved in cold ether. The ether solution was successively washed with ice cold water, cold saturated aqueous $NaHCO_3$ and again with ice cold water. After quickly drying over anhydrous Na_2SO_4, the azide solution was added in 20 min. to a cooled solution of L-proline ethylester (330 mg) in dry ether. After standing for 24 hours at 0°C, the solution was washed with dilute aqueous HCl to remove excess L-proline ethylester, and then with aqueous $NaHCO_3$, and finally with water.

Removal of the solvent under reduced pressure left a pale yellow solid of the carbobenzoxy-L-phenylalanyl-L-proline ethylester.

The whole carbobenzoxy compound dissolved in a mixture of 1 cc glacial acetic acid, 10 cc methanol and 2 cc water was hydrogenated in the presence of active Pd. black. After this procedure, the solvent was again evaporated to dryness under reduced pressure. The residue was then dissolved in dry methanol and saturated with ammonia under cooling.

After one hour, the methanol was evaporated off and the product recrystallized from ethylacetate-ligroin as fine needles: yield 0.28 g (36.8%), m.p. 127∼128°C, $[\alpha]_D^{20} = -100°$ (c, 1 in ethanol)

Anal. Found: C 68.91, H 6.53, N 11.63

Calcd. for $C_{14}H_{12}O_2N_2$: C 68.80, H 6.60, N 11.50

10. Growth test of rice seedlings.

Young seedlings of rice variety Norin 1, similar in height, were transferred to a Petri dish covered with 1% agar, containing various concentrations of compounds A, B, C, D, E and rosellinic acid.

The preparation was kept in a glass container at 30°C for 10 days (from June 18∼28, 1959) and the length of leaf and roots were measured.

The results are shown in the following Tables II and III.

Acknowledgement. The author wishes to express his sincere thanks to Prof. Y. Sumiki and Prof. H. Asuyama, Univ. of Tokyo, for their kind guidance and encouragement throughout this work. Thanks are also due to Mr. K. Kasai, Tea Division, National Tokai-Kinki Agr. Expt. Station, for supplying the culture of *Rosellinia necatrix*.

TABLE II. A COMPARISON OF THE RELATIVE LENGTH OF LEAF (%) OF RICE SEEDLINGS TREATED WITH VARIOUS CONCENTRATIONS OF COMPOUNDS A,B,C,D,E AND ROSELLINIC ACID (AVERAGE OF 20 SEEDLINGS)

		Concentrations							
Metabolites	Cont.	2.5×10^{-3}	5×10^{-3}	10×10^{-3}	4×10^{-4}	10×10^{-4}	2×10^{-5}	5×10^{-5}	10×10^{-5}
B	100	66.32	72.54	80.96	84.32	88.54	90.42	95.54	98.36
C	100	73.25	78.43	84.54	92.51	93.78	95.38	99.56	102.21
D	100	74.69	78.82	89.22	99.77	100.21	102.90	103.44	105.63
E	100	77.54	80.98	85.22	92.60	94.32	96.09	101.56	100.28
Rosellinic Acid	100	63.21	68.81	74.82	82.34	85.22	88.32	94.56	99.21

TABLE III. A COMPARISON OF THE RELATIVE TOTAL LENGTH OF ROOTS (%) OF RICE SEEDLINGS TREATED WITH VARIOUS CONCENTRATIONS OF COMPOUNDS A,B,C,D,E AND ROSELLINIC ACID (AVERAGE OF 20 SEEDLINGS)

		Concentrations							
Metabolites	Cont.	2.5×10^{-3}	5×10^{-3}	10×10^{-3}	4×10^{-4}	10×10^{-4}	2×10^{-5}	5×10^{-5}	10×10^{-5}
B	100	58.36	59.36	72.34	78.88	88.92	98.56	100.11	99.76
C	100	59.55	66.04	82.37	95.66	98.82	100.22	103.44	108.56
D	100	64.91	68.21	88.65	96.73	103.56	110.93	114.56	120.22
E	100	67.55	73.26	86.64	93.46	99.56	100.13	102.12	105.44
Rosellinic Acid	100	52.00	64.02	74.55	85.62	90.44	94.56	99.72	98.56

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 382~385, 1960]

Studies on the Degradation Mechanisms of Proteins and their Derivatives in the Foods

Part III. The Decompositions of Several Cysteine-aldehyde Compounds in Aqueous Solutions

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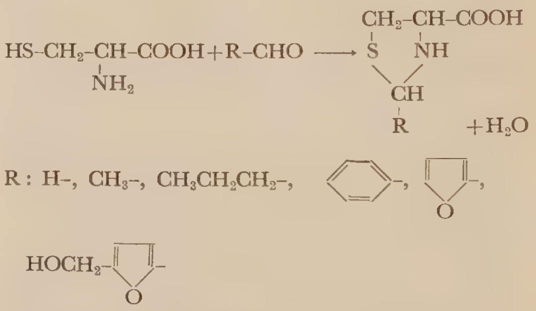
Cysteine-aldehyde compounds were prepared by the reactions of L-cysteine with formaldehyde, acetaldehyde, *n*-butyraldehyde, benzaldehyde and furfural in 50% ethanol solutions. Hydrogen sulfide and ammonia liberated from cysteine-aldehyde compounds in heated aqueous solutions (oil bath: 120°C) were determined. Although thiazolidine derivatives were stable generally in boiling aqueous solution, L-cysteine-furfural compound was unstable and a large amount of hydrogen sulfide compared with other compounds was released.

INTRODUCTION

In the previous paper[1] the authors pointed out that the blackening phenomenon of the can-wall of seasoning beef "Yamatoni" was due to the soy-bean meal hydrolysates (AMINOSAN-EKI) contained as the seasonings.

The heat labile sulfur compounds were pursued in AMINOSAN-EKI. The sulfur compounds were adsorbed on active carbon column and eluted with 0.1 N ammonium hydroxide. The concentrate of the elute remains intact in the reaction with *p*-chloromercuribenzoate in 0.33 M acetate buffer (pH 4.6) even after the concentrate was treated with tin-hydrochloric acid. It suggests that the presence of sulfhydryl group (-SH) or disulfide linkage (-S-S-) in the heat labile sulfur compounds is questionable[2].

In the present paper several thiazolidine derivatives were prepared from L-cysteine and aldehydes (formaldehyde, acetaldehyde, *n*-butyraldehyde, benzaldehyde, furfural and 5-hydroxymethylfurfural) in 50% ethanol[3].



These cysteine-aldehyde compounds except cysteine-5-hydroxymethyl-furfural compound were obtained in fine crystalline forms and their analytical values agreed with their theoretical ones.

The behaviors of these compounds in aqueous solutions heated on the oil bath (120°C) were investigated by the determinations of hydrogen sulfide[4] and ammonia[5].

EXPERIMENTAL

1. L-Cystine.

L-Cystine was prepared from human hair[6]. Neutralization was carried out with sodium acetate. Sixty-one grams of L-cystine were obtained from 700 g of washed and dried hair by the method of isoelectric precipitating recrystallization, $[\alpha]_D^{17} = -190.5°$.

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[1] Y. Obata, S. Sakamura, Y. Ishikawa and J. Mizutani, *J. Agr. Chem. Soc. Japan*, **31**, 585~7 (1957).

[2] Y. Obata, Y. Ishikawa and J. Mizutani, *J. Agr. Chem. Soc. Japan*, **34**, 336~339 (1960).

[3] M.P. Schubert, *J. Biol. Chem.*, **114**, 341~50 (1936).

[4] M.M. Brenner, J.L. Owades and R. Glyzniak, *Am. Brewer*, **87**, No. 3, 29~36 (1954).

[5] J.B. Sumner and G.F. Somers, *Laboratory Experiments in Biological Chemistry*, p. 67~71 (1949).

[6] S. Akabori and S. Mizushima, Chemistry of Protein (Kyoritsu Publishing Co. Ltd.), (1) p. 63~4 (1954).

2. L-Cysteine.

L-Cysteine was prepared by the method of reduction of L-cystine with tin-hydrochloric acid[7]. Seven grams of L-cysteine were yielded from 12 g of L-cystine.

3. Preparations of L-cysteine-aldehyde compounds.

L-Cysteine (8.3 m mol) was dissolved in 20 ml of 50% ethanol, aldehyde (8.3 m mol) added and the mixture was allowed to stand in an ice-box over night. Crystals were collected and recrystallized. These compounds gave negative nitroprusside reaction and gave yellow color with ninhydrin. Nitrogen was determined by the method of micro-Kjeldahl[5] and sulfur by alkali fusion[8].

a. L-Cysteine-formaldehyde compound.

Fine needles were obtained; yield 0.9 g (82%). Recrystallization was carried out from hot water with the addition of ethanol, m.p. 194~5°C (dec.).

Anal. Found: N, 10.71; S, 23.66%.
Calcd. for $C_4H_7NO_2S$: N, 10.52; S, 24.08%.

b. L-Cysteine-acetaldehyde compound.

Fine needles were obtained; yield 0.9 g (73%), m.p. 167~170°C (dec.).

Anal. Found: N, 9.14; S, 22.12%.
Calcd. for $C_5H_9NO_2S$: N, 9.52; S, 21.78%.

c. L-Cysteine-*n*-butyraldehyde compound.

Rectangular plates were obtained; yield 1.0 g (69%), m.p. 167~8° (dec.). They were recrystallized from hot water.

Anal. Found: N, 8.21; S, 18.60%.
Calcd. for $C_7H_{13}NO_2S$: N, 7.99; S, 18.30%.

d. L-Cysteine-benzaldehyde compound.

Rectangular plates were obtained; yield 1.3 g (77%). They were recrystallized from hot water, m.p. 159~160°C (dec.).

Anal. Found: N, 6.81; S, 15.27%.
Calcd. for $C_{10}H_{11}NO_2S$: N, 6.70; S, 15.32%.

e. L-Cysteine-furfural compound.

Almost colorless crystals were obtained; yield 1.1 g (66%). Recrystallization was carried out from hot ethanol, m.p. 128~9° (dec.).

Anal. Found: N, 6.89; S, 16.11%.
Calcd. for $C_8H_9NO_3S$: N, 7.03; S, 16.09%.

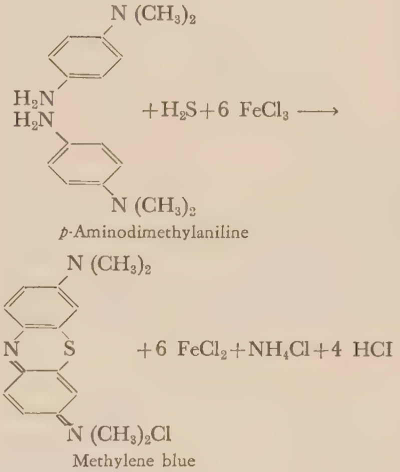
This compound was unstable very much and gradually changed into grayish brown, but decomposition point remained unchanged.

f. L-Cysteine-5-hydroxymethylfurfural compound.

5-Hydroxymethylfurfural was prepared by the method of R. Feulgen and K. Imhäuser[9]. Although it was treated as same as other cysteinealdehyde compound preparations, crystals were not obtained.

4. Determination of hydrogen sulfide.

Hydrogen sulfide was determined by the method of Brenner et al.[4]: concentration of methylene blue, which is formed by the reaction of hydrogen sulfide with *p*-aminodimethylaniline and ferric chloride, is measured colorimetrically.



a. Calibration curve.

Calibration curve for hydrogen sulfide determination (Fig. 1) was prepared from sodium sulfide. Concentration of sodium sulfide solution was determined iodometrically.

b. Apparatus.

The apprratus used is given in Fig. 2. Carbon dioxide gas current was used as a carrier of hydrogen ulfide sand the same results were obtained in the case of using nitrogen gas instead of carbon dioxide. Recovery of hydrogen sulfide of this apparatus was 95±5%.

c. Determining procedure.

Sample containing 32 mg of sulfur was taken in a flask and 10 ml of water were added. Passing through carbon dioxide, the solution was heated for thirty minutes, one, three and six hours respectively on the oil bath (120°C). After that, 10 ml of hydrochloric acid was added and carbon dioxide was passed through the reaction mixture furthermore thirty minutes. Hydrogen sulfide absorbed in 2% zinc acetate was derived into methylene blue and the absorbancy at 745 mμ was determined spectrophotometrically. The results are shown in Table I.

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[7] S. Akabori and S. Mizushima, Chemistry of Protein, (1) p. 389 (1954).

[8] K. Bailey, *Biochem., J.*, **31**, 1398 (1937).

[9] R. Feulgen and K. Imhäuser, *Z. physiol. Chem.*, **148**, 1~16 (1925).

5. Determination of ammonia.

Determination of ammonia was followed by the method of Nessler modified by Folin-Wu[5].

a. Calibration curve.

The standard graph was prepared by Nesslerizing ammonium sulfate solutions. The optical density of Nesslerizing solution at 420 mμ was read in the spectrophotometer. The calibration curve is given in Fig. 3.

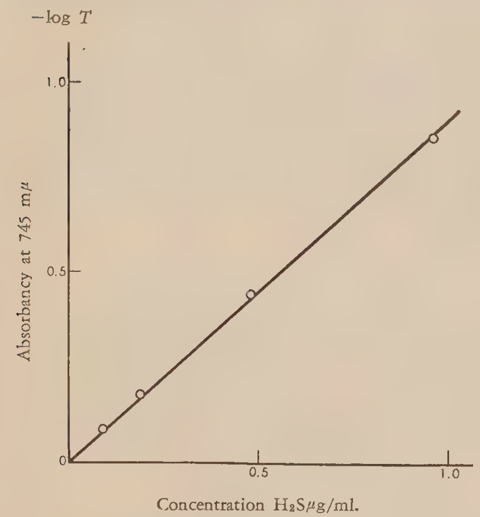

FIG. 1. Calibration curve for hydrogen sulfide determination.

TABLE I. DETERMINATIONS OF HYDROGEN SULFIDE LIBERATED FROM L-CYSTEINE-ALDEHYDE COMPOUNDS IN HEATED AQUEOUS SOLTIONS (Oil bath: 120°C)

Compound (Containing 32 mg S)	Hydrogen Sulfide (μg) Liberated after			
	0.5	1	3	6 Hours
L-Cystine	8.5	29	172	475
L-Cysteine	19	47	121	325
L-Cysteine-formaldehyde	10	20	90	264
L-Cysteine-acetaldehyde	8	19	84	232.5
L-Cysteine-*n*-butyraldehyde	—	7	29	97
L-Cysteine-benzaldehyde	7	15	60	195
L-Cysteine-furfural (I)	32	68	237	700
L-Cysteine-furfural (II)	106	195	723	—

L-Cysteine-furfural (II) was storaged for more than a month and considerably colored.

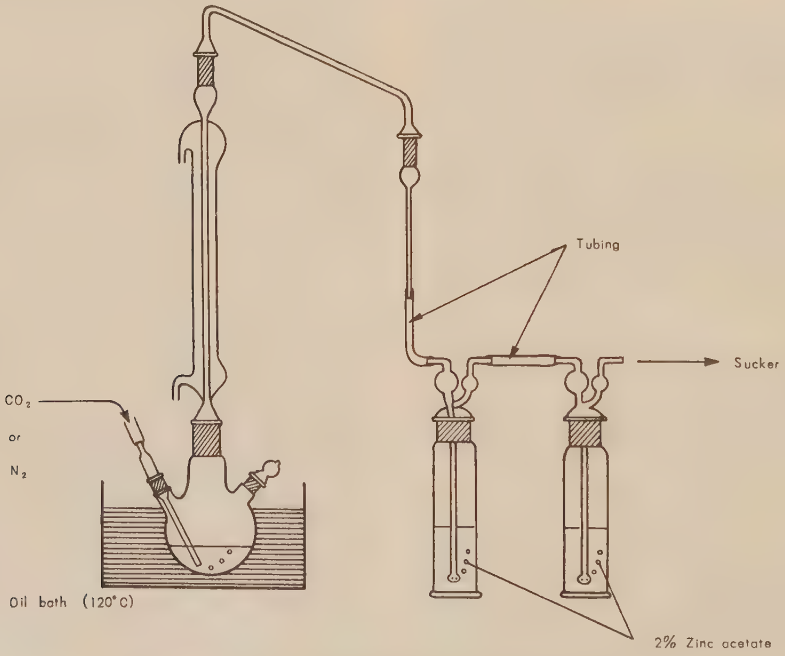

FIG. 2. Apparatus for hydrogen sulfide determination.

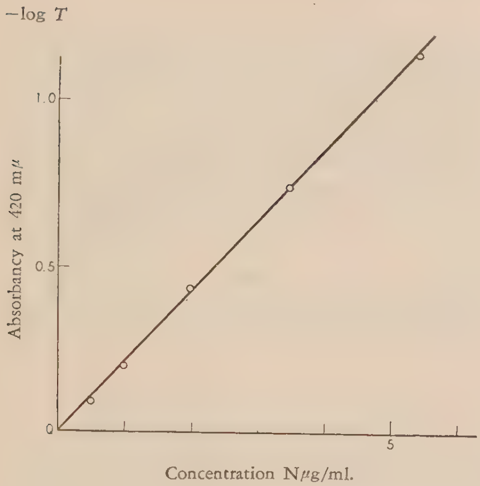

FIG. 3. Calibration curve for ammonia determination.

TABLE II. DETERMINATIONS OF AMMONIA LIBERATED FROM L-CYSTEINE-ALDEHYDE COMPOUNDS IN HEATED AQUEOUS SOLUTIONS (Oil bath : 120°C)

Compound (Containing 14 mg N)	Ammonia (μg) Liberated after 3	6 Hours
L-Cystine	99	160
L-Cysteine	127	228
L-Cysteine-formaldehyde	69	147
L-Cysteine-acetaldehyde	52	132
L-Cysteine-*n*-butyraldehyde	24	65
L-Cysteine-benzaldehyde	56	119.5
L-Cysteine-furfural	146	311

b. Apparatus.

The apparatus shown in Fig. 2 was applied. 0.1 N Hydrochloric acid was used as absorbing solution. Air which was free from ammonia by passing through dilute hydrochloric acid was employed and in the case of using nitrogen the results were the same.

c. Determining procedure.

A sample of the thiazolidine derivatives containing 14 mg of nitrogen was taken in a flask, 10 ml of water were added and the solution was heated on the oil bath (120°C) for three and six hours respectively. After the temperature of the oil bath went down below 80°C, 10 ml of 10% sodium hydroxide were added and furthermore thirty minutes aeration was continued. Ammonia absorbed in 0.1 N hydrochloric acid was determined by the method of Folin-Wu modified Nessler. Before the Folin-Wu modified Nessler solution was added, 1 ml of 10% potassium sodium tartrate per 50 ml was added in order to prevent the turbidity. The results of determinations are given in Table II.

DISCUSSION

Studies on the decomposition of cystine and cysteine in boiling aqueous solutions have already been carried out by J. I. Routh[10]. The present authors compared the heat instabilities of several cysteine-aldehyde compounds with cysteine and cystine.

L-Cysteine-aldehyde compounds were prepared by the reactions of L-cysteine with formaldehyde, acetaldehyde, *n*-butyraldehyde, benzaldehyde, furfural and 5-hydroxymethylfurfural in 50% ethanol. These compounds except L-cysteine-5-hydroxymethylfurfural were obtained in fine crystalline forms and their analyses agreed with their theoretical values.

The behaviors of these compounds in heated aqueous solutions were investigated by determining hydrogen sulfide and ammonia. As shown in Tables I and II, generally cysteine-aldehyde compounds were stable against the heat under the given condition so far as hydrogen sulfide and ammonia formation, but L-cysteine-furfural compound was unstable and large amounts of hydrogen sulfide and ammonia were liberated. This tendency was increased with storage of this compound. Other thiazolidine derivatives also have such tendencies though it is weaker than cysteine-furfural compound.

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[10] J.I. Routh, *J. Biol. Chem.*, **126**, 147~54 (1938) ; *ibid.*, **130**, 297~304 (1939).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 386~396, 1960]

Studies on Osmophilic Yeasts

Part X. Influence of the Environmental Factors on the Change of Microflora during the Ripening Process of Soy-mash

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Influences of concentrations of sodium chloride and pH values of soy mash on the changes of microflora during the ripening process were investigated. The proportions of various yeasts in the soy mash were proved to be changed with the above two environmental factors. And it was shown that growth sequence of yeasts in soy mash was controlled according to their pH sensitivities at the hypertonic condition. From young soy mash (3 days' old), the following yeasts were isolated: salt-tolerant type....*Saccharomyces rouxii* Boutroux, *Torulopsis famata* (Harrison) Lodder et Kregervan Rij, *Candida polymorpha* Ohara et Nonomura, *Pichia farinosa* (Lindner) Hansen, and *Trichosporon behrendii* Lodder et Kreger-van Rij; salt-sensitive type....*Candida tropicalis* (de Beurm., Gougerot et Vaucher) Ota and *Candida rugosa* (Anderson) Diddens et Lodder.

INTRODUCTION

Few papers have appeared which describe the ecological studies on the change of microflora during the ripening process of soy-mash.

Takahashi and Yukawa[1] stated that *Zygosacch. soya* was isolated from the young mash, whereas from the mature mash *Zygosacch. majar* was found. Kita[2] found that the species of yeasts isolated from the mash which had passed through the fermentation period in summer were quite different from those found in the immature mash before summer. Yoshii and Nakano[3] investigated the changes of microflora during the soy-brewing in terms of the plating counts of total yeasts, film-forming yeasts, total bacteria, bacilli and the lactic acid bacteria.

The soy mash differs significantly from other brew products in its high content of sodium chloride (about 18%) and high nitrogen concentrations (total nitrogen 1.0~1.5%). It is assumed that such a special environment has a remarkable effect on the microflora of the soy mash. The physiological aspects of osmophilic yeasts in the environment of high concentrations of sodium chloride were described previously by the author[4,5].

As far as the various soy mashes in the commercial scale (of the factory) were concerned, it was found that the pH values gradually shifted during the ripening process. The pH of the mash prepared in winter or spring was 6.0~6.6 and decreased to 4.4 in the case of fermentation period in summer. On the other hand, the initial pH value of the mash which was prepared in summer was 5.0~5.4 and rapidly became to 4.4.

When the mash was placed at 30°C which was suitable temperature for the growth of usual microorganisms, the pH decreased from 6.0~6.6 to below 5.0 in 15 days.

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[1] T. Takahashi and M. Yukawa, *Memoirs of the College of Agr., Tokyo Imp. Uuiv.*, **5**, 227 (1915).

[2] G. Kita, *J. Chem. Ind. Tokyo, Japan*, **14**, 109 (1911).

[3] H. Yoshii and M. Nakano, *J. Fermentation Technol.*, **34**, 361 (1956).

[4] H. Ônishi, This Bulletin, **21**, 143 (1957).

[5] H. Ônishi, This Bulletin, **23**, 351 (1959).

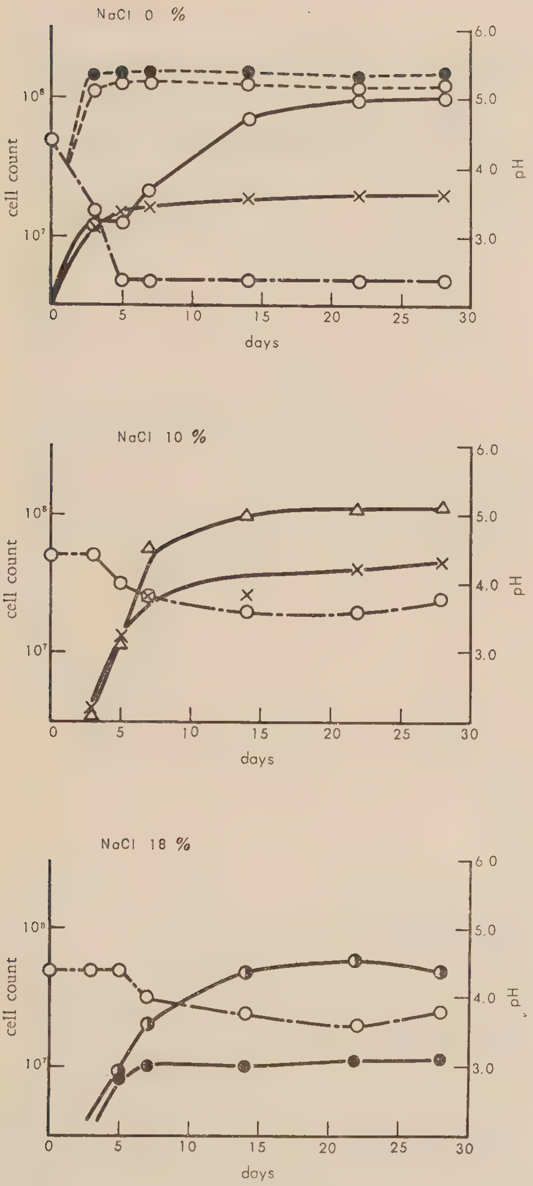

FIG. 1. Changes of Microflora of Young Soy Mash in Media of Various Concentrations of Sodium Chloride. (1) pH 4.4 koji extract.

Bacteria { Rod : ○----○
Cocci : ●----●

Yeast { Type I: ○—○
Type II: ×—×
Type III: △—△
Type IV: ●—●
Type V: ◐—◐

pH: ○—·—·—·—○

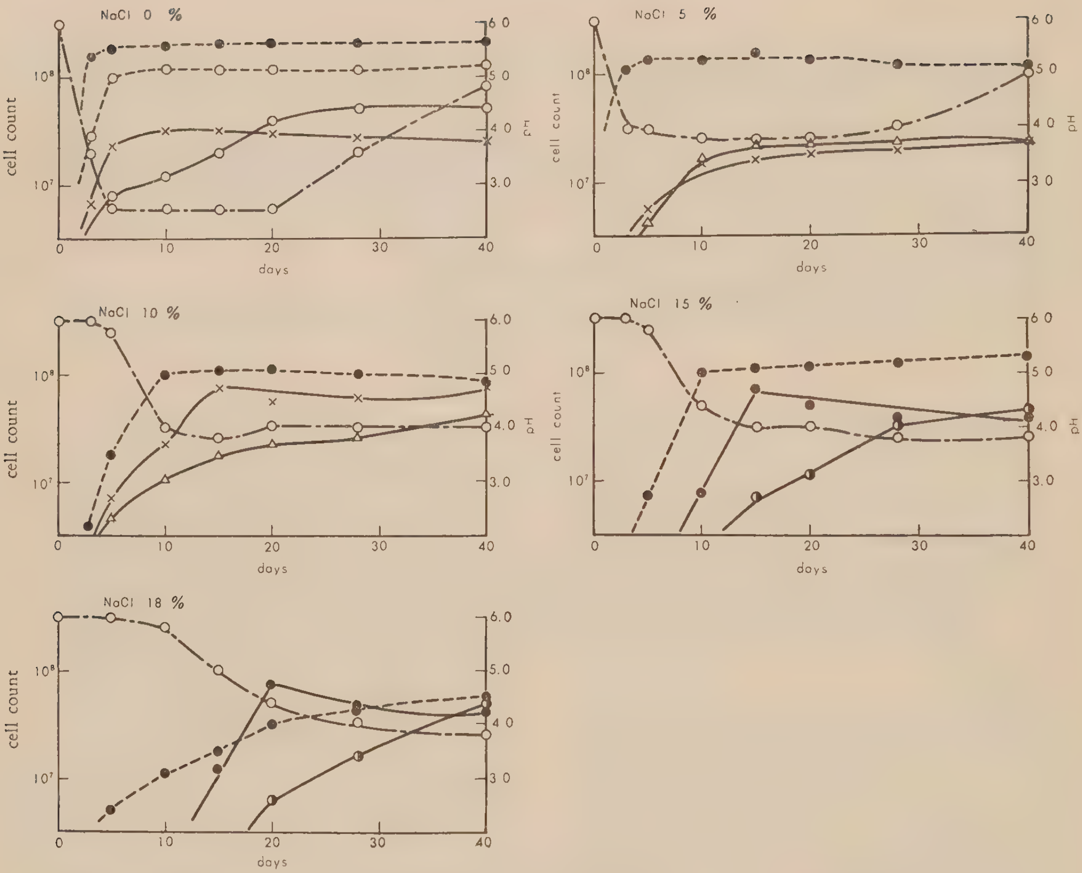

FIG. 2. Changes of Microflora of Young Soy Mash in Media of Various Concentration of Sodium Chloride. (2) pH 6.0 koji extract.

Bacteria { Rod : ○----○
Cocci : ●----●

Yeast
Type I : ○—○
Type II : ×—×
Type III : △—△
Type IV : ●—●
Type V : ◑—◑

pH : ○—·—·—·—○

As described in the previous paper[4], the growth in the NaCl-free medium of *Sacch. rouxii*, which played an important role in ripening soy mash, was allowed at the wide range of pH (3.0～8.0), while in the 18% NaCl medium, the yeast grew only at pH 4.0～5.0. From these facts, it was estimated that an important factor controlling the change of microflora during the soy-brewing might be the pH value of the mash.

Various salt-sensitive yeasts and bacteria may exist in the soy mash at the initial stage of brining as well as the salt-tolerant microorganisms. It may be interesting to see the interaction and the selection among these in the presence of various concentration of sodium chloride at different pH values.

In the present paper, the influences of various environmental factors on the change of microflora in the soy mash are described. However, the attention is focused to the pH value and

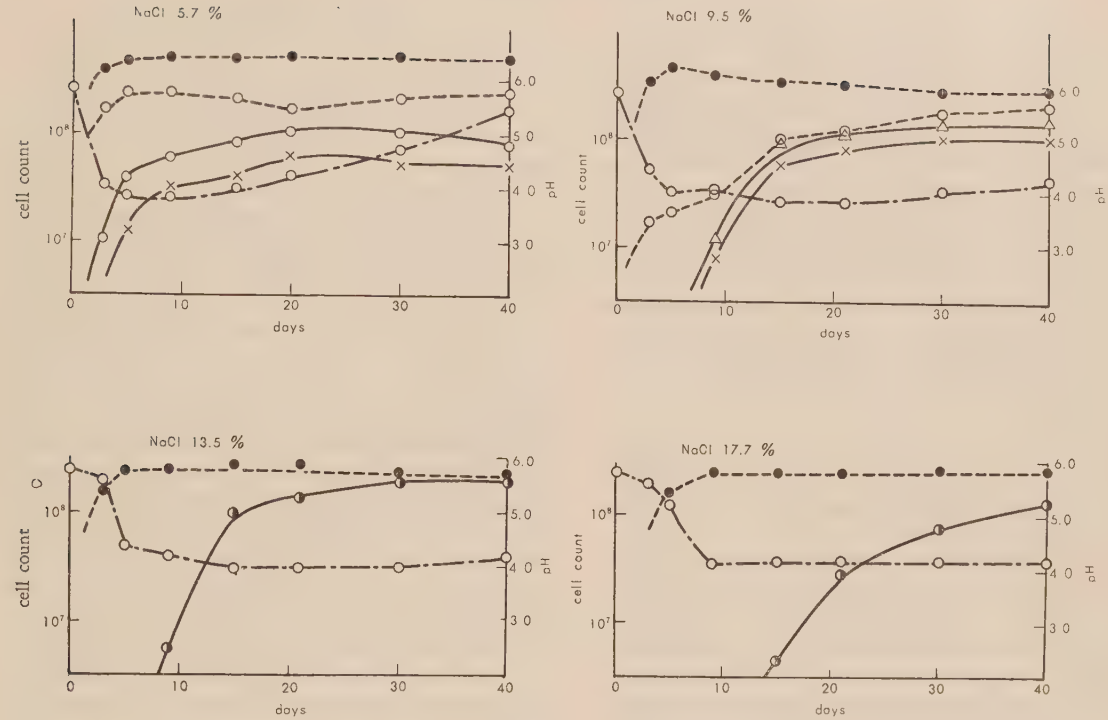

FIG. 3. Changes of Microflora in Soy Mashes of Various Concentrations of Sodium Chloride.

Bacteaia { Rod : ○----○
Cocci : ●----●

Yeast
Type I: ○—○
Type II: ×—×
Type III: △—△
Type IV: ●—●
Type V: ◐—◐

pH: ○—·—·—·—○

the salt concentration which have seemed important among the environmental factors.

MATERIALS AND METHODS

Investigation on the microflora was carried out by means of the routine techniques in the elective culture method and the enrichment method in soil microbiology[6].

Media: Koji extract (Balling 10°) were used which contained sodium chloride at various concentrations and of which the pH values were modified.

Materials: Samples of soy mashes were taken from the industrial tanks of the Noda Shoyu Co. One spoonful of the mash was suspended in 100 ml sterile saline prior to the examination.

Method of cultivation and observation: One to two drops of the mash suspension were inoculated into 100 ml of the koji-extract media and incubated at 30°C. Enumeration and identification of yeasts and bacteria were conducted under the microscope using the Thoma's Haemacytometer. The strains of yeasts were purified through single colony isolation and served for the taxonomic study.

EXPERIMENTALS

[I] Influences of the various concentrations of sodium chloride and of the different pH values on the changes of microflora in the soy mash.

The samples served for the following studies were taken from the young soy mash of 3 days' old after brining in August. This season was the fermentation period of the soy mash.

(1) Change of microflora in koji extract media of initial pH 4.4 in the presence of various concentrations of sodium chloride. (Fig. 1)

When the medium was free of NaCl, the pH of the

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6) S.A. Waksman, Soil Microbiology, p. 50 (1952).

medium went down to 2.4 within 5 days. The shift of pH value was probably due to the growth of acid-forming cocci and rods. Approximately 10^9 of these bacteria were found in 1 ml of the medium. As for the yeasts, Type I and II were predominating and the former type propagated forming luxuriant pellicles on the whole surface of the medium. On the contrary, the presence of 5% or more NaCl in the medium did not permit the growth of bacteria and consequently the pH remained around 4.0. The slight decrease of pH values seemed to be due to the growth of yeasts. In NaCl-containing media, the different types of yeasts developed according to the concentrations of the salts: types I and II were predominating in the 5% NaCl medium, types I, II and III in 10% NaCl, and types IV and V in 15% or more NaCl.

(2) Change of microflora in koji extract medium of initial pH 6.0 in the presence of various concentrations of sodium chloride. (Fig. 2)

In the absence of NaCl, the growth of microorganisms and the fall of the pH value were similar to the results described in the preceeding section (1). However, the results were quite different when the medium contained NaCl. The media allowed the appreciable growth of acid-forming cocci in the early period of incubation, and the yeasts appeared later on. In the 18% NaCl medium, yeasts grew after pH of the medium fell below 5.0 in the following sequence: type IV appeared in the first place and then *Sacch. rouxii* of type V began to grow, the latter getting predominant finally.

In conclusion, rod-shaped bacteria were generally salt-sensitive and could not grow in a saline medium containing more than 10% NaCl, whereas cocci were considerably salt-tolerant. In the saline media, however, pH of the medium markedly influenced the growth of cocci. Namely, cocci could not grow in the saline medium of low pH value such as 4.4. (c. f.: Sakaguchi[7])

Yeasts were able to grow rapidly in the saline medium of pH 4.4, but when the pH was 6.0, the growth of yeasts could not start until the pH fell below 5.0. In the latter case, the yeasts of different types appeared in sequence as stated above.

(3) Change of microflora in the soy mashes of the various concentrations of sodium chloride. (Fig. 3)

Change of microflora in the soy mashes with the various NaCl concentrations was investigated. As shown in Fig. 3, cocci developed vigorously irrespective of the NaCl concentrations and consequently the pH of mashes went down. Rod-shaped bacteria did not grow in the soy mashes where the NaCl concentrations were more than 13.5%.

The sequences in the growth of various types of yeasts were similar to those described in the preceeding section (2).

(4) Analysis of the koji-broth fermented at various conditions described in the section (1) and (2).

It was assumed that the products by the different microflora would be dissimilar. Therefore, residual sugars and alcohol production in the modified koji extract media incubated with soy mashes were assayed after 35 days' incubation. The conditions of the experiments were described in the section (1) and (2). The amount of alcohol produced in the medium containing 10% or less NaCl was lesser than that expected on the basis of consumed sugar (Table I). The loss of alcohol might be due to the dissimilation by the pellicle-forming yeasts of type I and III. On the other hand, much alcohol was recovered in the 18% NaCl medium. The fermented medium containing 10% or less NaCl had characteristic odor of ethylacetate or mouldy smell which is not preferable for soy sauce. It should be noticed that NaCl at various concentrations controlling the change of microflora modifies the flavor and taste of soy sauce.

TABLE I. ANALYTICAL DATA OF THE FERMENTED BROTH IN EXPERIMENT (1) AND (2).

Initial pH of media	NaCl concentrations of media	Reducing sugars (as glucose) %	ethanol %
4.4	0%	0.91	0.05
	5	0.98	0.40
	10	0.96	0.01
	15	0.97	1.30
	18	1.00	1.19
	0%(Control)	8.77	0
6.0	0%	0.75	0.04
	5	0.63	0.66
	10	2.02	0.27
	15	1.07	0.95
	18	0.88	1.07
	0%(Control)	8.38	0.01

[II] Characteristics of Microflora in the Old Soy Mash.

The number of viable yeast cells in the soy mash of the fermentation period in summer were estimated to be 10^7 per ml of mash by plating method using koji agar containing 18% NaCl. In this period *Sacch. rouxii* was predominant.

In the older soy mash elapsed more than one year after brining, viable yeast cells were 10^4 per ml of mash and the predominant type of yeasts belonged to genus *Torulopsis*. This type of yeasts did not grow in the

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[7] K. Sakaguchi, This Bulletin, **23**, 22 (1959).

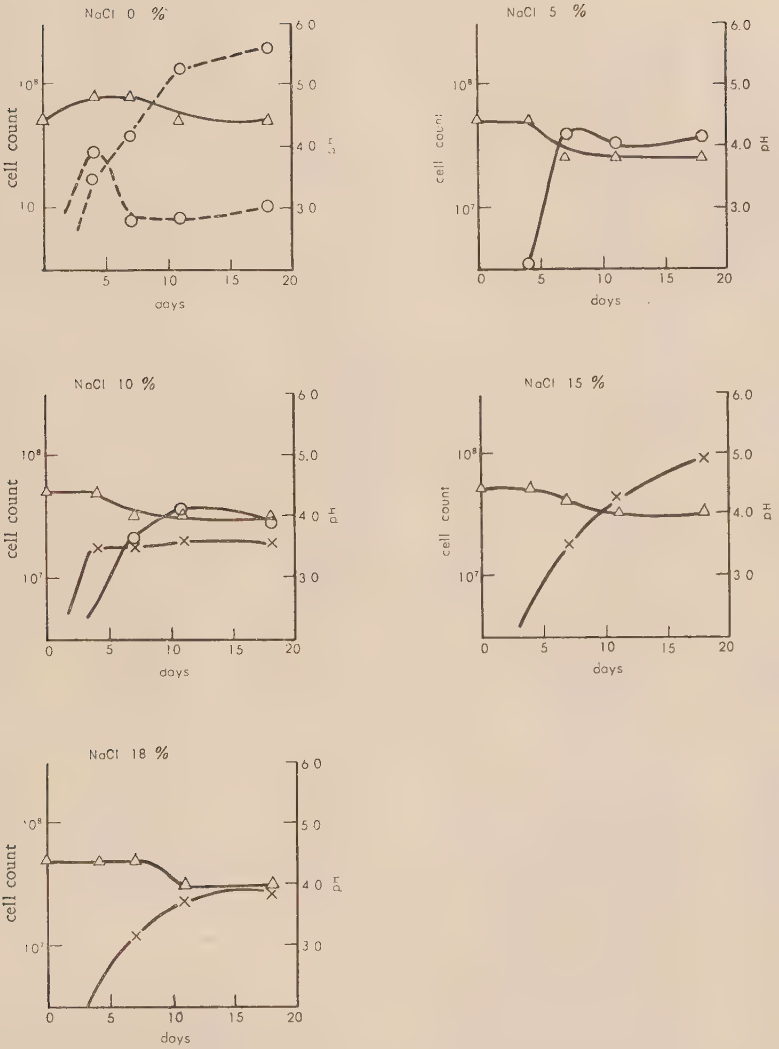

FIG. 4. Changes of Microflora of Old Soy Mash in Media of Various Concentrations of Sodium Chloride. (1) pH 4.4 koji extract.

Bacteria { Rod : ○----○
Cocci : ●----●
Yeast { Big cells : ○——○
Small cells : ×——×
pH : △——△

NaCl-free medium. When the old soy mash was inoculated into pH 4.4 or 6.4 koji extract containing NaCl at various concentrations, the microflora changed as illustrated in Figs. 4 and 5.

In the NaCl-free medium, both cocci and rod-shaped bacteria grew slowly whereas yeasts could not grow, and in the saline medium, vice versa. The yeasts grown in the saline medium were small and oval-shaped and could not grow in the NaCl-free medium. This yeast seemed to have obligate halophilic property. Microbiological properties of this yeasts were studied in detail and reported elsewhere[8].

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[8] H. Ônishi, This Bulletin, **24**, 226 (1960).

TABLE II. MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE ISOLATED YEASTS.

		Type I *Candida krusei* (Cast.) Berkhout	Type II *Candida tropicalis* (Cast.) Berkhout	Type III *Pichia farinosa* (Lindner) Hansen	Type IV *Torulopsis famata* (Harrison) Lodder et Kreger-van Rij
Vegetative cells (after 5 days' cultivation in koji extract at 30°C)		Cells are usually ellipsoidal to elongate. They measure about $(3\sim5)\times(6\sim12)\ \mu$. A dry white pellicle is formed.	Cells are usually round or oval, $5\sim10\ \mu$. A sediment and a ring are formed, occasionally islets.	Cells are oval to cylindrical $(4\sim6)\times(4\sim8)\ \mu$. A wrinkled pellicle is formed.	Cells are small, oval, $(2\sim4)\times(4\sim7)\ \mu$. The cells are single, in pairs or clinging together in short chains. A sediment and a ring are formed, occasionally islets.
Growth on koji agar, at 30°C		After one month, the color of the streak culture is grey to yellow. The streak culture is dull, flat usually smooth or delicately wrinkled. The margin is sinuous with distinct pseudomycelium fringes.	After one month, color is cream to yellowish. The appearance of the streak culture is dull to somewhat glistening, folded. The margin is surrounded by mycelium	After one month, the streak culture is grey, dull, flat and wrinkled.	After one month, the streak culture is cream to grey, usually smooth, slightly glistening.
Slide culture on potato agar		Rich development of pseudomycelium.	Rich development of pseudomycelium	Primitive or no pseudomycelium	No pseudomycelium
Ascospore formation		not formed	not formed	Spores are formed after heterogamous conjugation. The spores are round, $2\sim4$ per ascus.	Not formed
Fermentation of sugars	Fermented	glucose	glucose, galactose, maltose, saccharose	glucose, galactose	no fermentation
	Unfermented	galactose, maltose, saccharose, lactose	lactose	maltose, saccharose, lactose	glucose, galactose, maltose, saccharose, lactose
Assimilation	Assimilated	glucose	glucose, galactose, maltose, saccharose	glucose, galactose	glucose, galactose, maltose, saccharose
	Not assimilated	galactose, maltose, saccharose, lactose	lactose	maltose, saccharose, lactose	lactose
Assimilation of Nitrate		Negative	Negative	Negative	Negative
Growth in ethanol		growth	growth	growth	no growth
Splitting of arbutin		Negative	Slightly positive	Positive	Negative
Salt-tolerance		13% NaCl: growth 15% NaCl: no growth	13% NaCl: growth 15% NaCl: no growth	18% NaCl: growth	18% NaCl: growth

		Type V	Type a	Type b	Type c	Type d
		Saccharomyces rouxii Boutroux	*Trichosporon behrendii* Lodder et Kreger-van Rij	*Candida rugosa* (Anderson) Diddens et Lodder	*Trichosporon cutaneum* (de Beurm., Gongerot et Vaucher) Ota	*Candida polymorpha* Ohara et Nonomura
Vegetative cells (after 5 days' cultivation in koji extract at 30°C)		Cells are round or short oval, single or in pairs. The cells usually measure $(5\sim8)\times(5\sim10)\ \mu$. A sediment and a ring are formed.	Cells are oval, $(3\sim5)\times(4\sim6)\ \mu$, some cells are elongated. Islets are formed.	Cells are oval or ellipsoidal, $(2\sim4)\times(4\sim8)\ \mu$. A thin pellicle is formed.	Cells are round, oval, ellipsoidal to elongated, $(4\sim5)\times(4\sim20)\ \mu$. A grey wrinkled pellicle is formed.	Cells are bottle shaped triangular or ellipsoidal $(3\sim4)\times(6\sim8)\ \mu$. Thin islets are formed.
Growth on koji agar, at 30°C		After 2 weeks, the streak culture is light cream colored, dull glistening, raised and almost smooth.	After one month, the streak culture is yellowish-white, tough, raised, wrinkled and hairy.	After one month, the streak culture is grey-yellowish, dull and wrinkled.	After one month the streak culture is yellowish, grey, raised, wrinkled, dry, dull, hairy.	After one month, the streak culture is grey-yellowish, glistening, smooth and flat.
Slide culture on potato agar		No pseudomycelium	True mycelium with blastospores and arthrospores is formed.	Well-developed pseudomycelium	True mycelium with blastospores and arthrospores is formed	Pseudomycelium is not much developed.
Ascospore formation			not formed	not formed	not formed	not formed
Fermentation of sugars	Fermented	glucose, maltose	glucose, galactose, maltose, saccharose, raffinose	no fermentation	no fermentation	no fermentation
	Unfermented	galactose, saccharose, lactose	lactose	glucose, galactose, maltose, saccharose, lactose	glucose, galactose, maltose, sacchorose, lactose	glucose, galactose, maltose, saccharose, lactose
Assimilation	Assimilated	glucose, galactose, maltose	glucose, galactose, maltose, saccharose	glucose, galactose	glucose, galactose, saccharose, maltose	glucose, galactose, saccharose, maltose
	Not assimilated	saccharose, lactose	lactose	maltose, saccharose, lactose	lactose	lactose
Assimilation of Nitrate		Negative	Negative	Negative	Negative	Negative
Growth in ethanol		no growth	growth	growth	no growth	no growth
Splitting of arbutin		Negative	Positive	Very slightly positive	Very slightly positive	Positive
Salt-tolerance		18% NaCl : growth	18% NaCl : growth	13% NaCl : growth 15% NaCl : no growth	13% NaCl: growth 15% NaCl: no growth	18% NaCl: growth

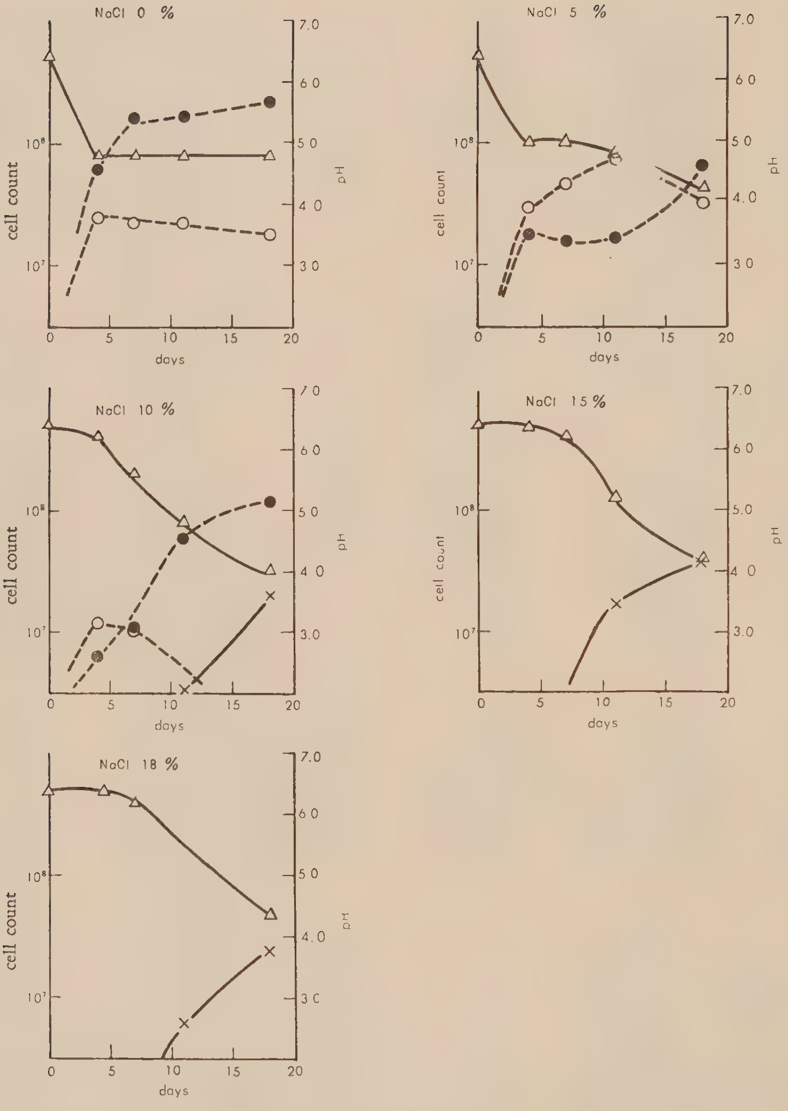

FIG. 5. Changes of Microflora Old Soy Mash in Media of Various Concentrations of Sodium Chloride. (2) pH 6.4 koji extract.

Bacteria { Rod : ○----○
Cocci : ●----●
Yeast { Big cells : ○——○
Small cells : ×——×
pH : △——△

TABLE III. INFLUENCES OF THE pH VALUES OF MEDIA ON THE GROWTH OF SALT-TOLERANT YEASTS

yeasts		*Torulopsis famata*		*Candida polymorpha*		*Sacch. rouxii* (isolated)		*Sacch. rouxii* (stocked)	
Media	incubation period	6 days' Incubation	14 days' Incubation	6 days	14 days	6 days	14 days	6 days	14 days
NaCl-free	pH 3.0	−	±	−	−	+++	+++	+++	+++
〃	pH 4.0	+	++	+	+	+++	+++	+++	+++
〃	pH 5.0	++	+++	++	+++	+++	+++	+++	+++
〃	pH 6.2	++	+++	++	+++	+++	+++	+++	+++
〃	pH 7.0	++	+++	++	+++	+++	+++	+++	+++
NaCl 18%	pH 3.0	−	−	−	−	++	++	++	++
〃	pH 3.8	−	+	−	+	+++	+++	+++	+++
〃	pH 5.0	+	+++	±	++	++	+++	++	+++
〃	pH 6.0	±	+++	±	++	−	++	−	−
〃	pH 7.0	−	++	−	+	−	−	−	−

[III] Taxonomic Studies on the Isolated Yeasts.

The yeasts arisen from the previous experiments, which were described in chapter [I], were purely isolated and classified according to Lodder and Kreger-van Rij[9]. Microbiological properties of these yeasts were shown in Table II. Types I∼V appeared always in soy mash and types a∼d were occasional.

[IV] Relationships between the Growth Sequence of Yeasts and pH Values in the Soy Mash.

Torulopsis famata of type IV, *Candida polymorpha* of type d and *Saccharomyces rouxii* of type V had a highly salt-tolerant property and were found in the young soy mashes.

In the medium containing large amounts of NaCl, these yeasts grew in a constant sequence as stated above, i. e. first, type IV and then type V. It was examined whether this sequential growth was attributed to the pH-sensitivities of yeasts at the high concentration of NaCl. Koji extract media of various pH values and the media supplemented with 18% NaCl were employed for the study. One loopful of the yeast taken from the fresh culture on koji extract agar was suspended in sterile saline and one drop of this suspension was inoculated into each medium. The medium was incubated at 30°C. As shown in Table III, *Sacch. rouxii* (stock culture) and type V yeast were able to grow well in the wide range of pH 3∼7 when the media were NaCl-free, although in the 18% NaCl media, their growth was not allowed at the neutral side. On the other hand, type IV and d yeasts could not grow at pH 3.0 and their optimal growth, irrespective of the presence of NaCl, was exhibited at the relatively neutral conditions, pH 5∼7. These results may explain well the above described observation, that is, the pH value of the soy mash is an important factor which controls the sequence of yeast growth.

DISCUSSION

Yoshii and Nakano[3] reported that number of viable cells of lactic acid bacteria in soy mash decreased with the advance of ripening from 10^8 per ml at the initial stage of brining to 10^4∼10^5 per ml at the fermenting period. This fact is consistent with the author's observation that acid-forming cocci can not grow in a 18% NaCl medium at low pH such as 4.4 although the cocci grow well in the similar medium at pH 6.0. After acid-forming cocci have propagated in young soy mash of pH 6.0 lowering the pH below 5.0, yeasts start to grow vigorously.

Thus, principal roles of acid-forming cocci in young soy mash are to produce lactic acid at the initial stage of ripening of soy mash, and to lower the pH value of mash consequently bringing about a suitable environment for growth and fermentation of yeasts. Hori et al.[10] reported that principal organic acid in soy-sauce is lactic acid and that acetic or succinic acid is minor.

Yeast flora is clearly controlled by the concentrations of NaCl in the mash according to the salt-tolerant properties of individials. As the saltsensitive yeasts are able to grow in the medium of 0∼10% NaCl much faster than the salt-tolerant yeasts, the formers predominate over the latter in the mash containing relatively low concentrations of NaCl. In the medium of high concentrations of NaCl such as 18%, growth sequence of yeasts is controlled by pH values of the medium. When the brewing is initiated at pH 4.4, *Sacch. rouxii* can grow vigorously and quickly, while in the case where the pH is decreasing gradually from 6.0 to 5.0, the growth of *Torulopsis* preceed *Sacch. rouxii*, and the latter predominates finally playing an important role in soy ripening.

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[9] J. Lodder and M.J.W. Kreger-van Rij, The Yeasts, a taxonomic study (1952).

[10] S. Hori et al., *J. Agr. Chem. Soc. Japan*, **31**, 22 (1957).

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtedness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. I also wish to thank Dr. M. Mogi, the Director of Noda Institute for Scientific Research, for his encouragement and Mr. A. Kaneko for his willing help at all stages of this work.

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Biochemical Studies on the Formation of the Silkprotein

Part IX. The Direct and Indirect Formation of the Silkprotein during the Growth of the Silkworm Larva

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Received October 14, 1959

A large part, about 70 per cent, of the silkproteins produced by one silkworm is directly derived from the proteins of the mulberry leaves (direct formation), but some part of the silkproteins, about 30 per cent, is also derived from the tissue proteins and body fluid proteins, in relation to the metamorphosis of the silkworm larva (indirect formation); there are two pathways in the formation of the silkproteins during the growth of the silkworm larva, i.e., direct formation and indirect formation.

Biosynthesis of the silkproteins in silkworms has been studied by a few research workers[1~3], but little is known of the formation of the silkproteins in the course of the silkworm growth. The author has studied, from a biochemical standpoint, the formation of the silkproteins during growth of the silkworm larva, and has recently found that a large part of the silkproteins produced by one silkworm, about 70 per cent., is directly derived from the proteins

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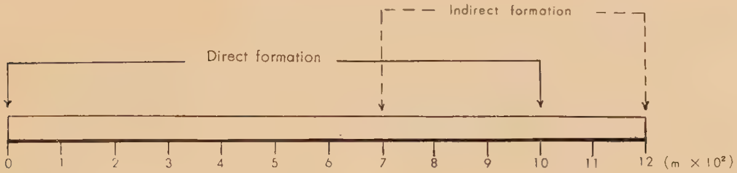
Some parts of this study were stated at the IV International Congress on Biochemistry held in Vienna, September, 1958.

1) T. Fukuda, J. Kirimura, M. Matuda and T. Suzuki, *J. Biochem.*, **42**, 341 (1955).

2) K. Shimura, *J. Japanese Biochem.* (*Seikagaku*), **28**, 197 (1956).

3) Y. Miura, H. Itoh and S. Takeyama, Intern. Sym. Enzyme Chem., Tokyo (1957).



Length of a silk filament unwound from one cocoon

FIG. 1. Direct formation and indirect formation of the silk

of the mulberry leaves (direct formation), but some part of the silkproteins, about 30 per cent., is also derived from the tissue proteins and body fluid proteins, in relation to the metamorphosis of the silkworm larva (indirect formation). It has also been clarified in the present study that the direct formation of the silkproteins takes place during the period between the 4th day of the 5th instar and maturity, but the indirect formation takes place during the period from the 8th day of the 5th instar to just before termination of the spinning, after reaching maturity.* In conclusion, it is shown in the present study that there are two pathways in the silkprotein during growth of the silkworm larva, i. e., the direct formation and indirect formation (Fig. 1).

After mulberry leaves labeled with C^{14} or amino acids labeled with C^{14} were given per os to the silkworm larva in the different stages, these silkworms were reared on non-radioactive mulberry leaves until they produced their cocoons. These cocoon fibres were unwound, and the distribution of C^{14} in each silk filament, the length of which was about 1,200 m, was investigated.

If the silkproteins are directly derived from amino acids of the mulberry leaves, it would be expected that a great amount of C^{14} given to the silkworm would appear concentrated to a certain extent in the silk filament unwound from one cocoon, and also its location would move regularly on the silk filament, parallel to the time when the radioactive mulberry leaves or the radioactive amino acids are given to the silkworm. If part of the silkproteins is also derived from proteins of the tissues, such as the alimentary canal, the muscles, etc., in relation to the metamorphosis of the silkworm larva, it would be expected that C^{14} would appear on each silk filament, in common with all the silkworms, to a certain extent from the middle to the rear, regardless of the time when the radioactive mulberry leaves or the radioactive amino acids are given to the silkworms. The present study is carried out to clarify this point.

EXPERIMENT

1) Preparation of the Radioactive Mulberry Leaves

The radioactive mulberry leaves were prepared by placing a mulberry tree for 5 hours in an atmosphere containing $C^{14}O_2$. The radioactivity of these leaves was 4,134 count/sec./cm². A radioautograph obtained by superposition of an X-ray film on the chromatogram of the acid hydrolysates of the radioactive mulberry leaves showed that the amino acids, such as glycine, serine, glutamic acid, aspartic acid, leucine, phenylalanine, tyrosine, valine, proline, lysine etc. contained C^{14} in their molecules (Fig. 2). This fact shows that the radioactivity of the mulberry leaves is due to the existence of the C^{14} in the molecules of the constituent amino acids of the mulberry leaves.

2) The Amino Acids labeled with C^{14}

The radioactive amino acids used in the present study were glycine-1-C^{14}, glycine-2-C^{14}, L-serine all labeled with C^{14} and DL-alanine-1-C^{14}. These amino acids were obtained from the Radiochemical Centre, Amersham, England.

3) Administration of the Radioactive Mulberry Leaves and the Radioactive Amino Acids to the Silkworms.

After 1 cm² per larva of the mulberry leaves labeled with C^{14} and 0.5 μc per larva of the amino acids labeled

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* "Maturity" corresponds to the moment when silkworms stop eating mulberry leaves at the end of the 5th instar, i.e., about one day before spinning.

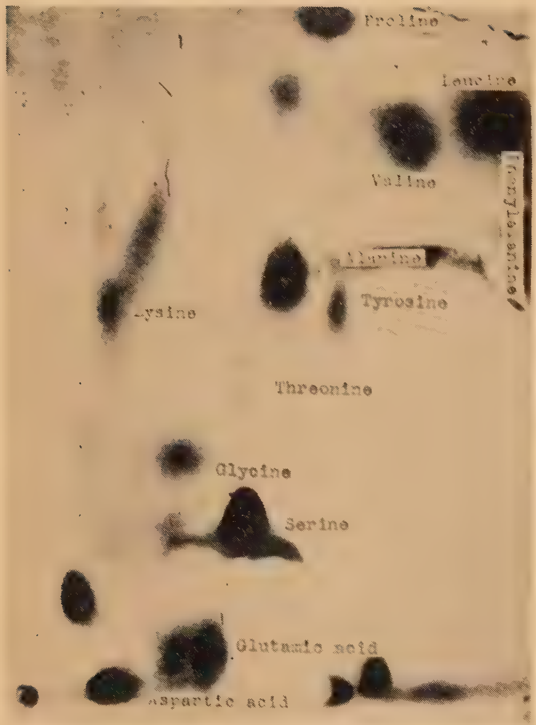

FIG. 2. Radioautograph of the chromatogram of the acid hydrolysates of the radioactive mulberry leaves

with C^{14} were given per os to the silkworms, Nichi 122×Si 115, in different stages from the beginning of the 5th instar to the end of the 5th instar, these silkworms were reared on normal mulberry leaves until they produced their cocoons.

4) Distribution of C^{14} on the Silk Filament

All the cocoons produced by the silkworms given 1 cm^2 per larva of the radioactive leaves and 0.5 μc per larva of the radioactive amino acids in different stages were unwound with an instrument, and each silk filament obtained from the cocoons was cut into length of 112.5 m, and each piece was degummed by boiling repeatedly in distilled water and M/50 sodium carbonate solution. Each length was washed with ethanol and ether, and dried. Each part obtained from the silk filament was rearranged on a sheet in the order obtained from one cocoon. A radioautograph of the silk was obtained by superposition of an X-ray film on the sheet for about 15 days (Fig. 3).

5) Radioactivity of the Amino Acids isolated from the Radioactive Silk.

a) The silk produced by the silkworm given the radioactive mulberry leaves.

The fibroin and the sericin were separated, according to the procedure described in a previous paper (4), from the radioactive cocoon fibres produced by the silkworms having consumed the radioactive mulberry leaves at the 4th day of the 5th instar. 80 mg of the fibroin was hydrolyzed with 4 ml of 12 N hydrochloric acid for 6 hours as usual. After the hydrolysate was freed of excess hydrochloric acid by repeated evaporation, the residue was analyzed by paper chromatography, using phenol containing ammonia and *n*-butanol-acetic acid saturated with water. After the location of the amino acids on the chromatogram obtained was checked with ninhydrin solution, the radioactivity of each amino acid was measured with an SC-16 Windowless Gas Flow Counter (Table I).

b) The silk produced by the silkworm given glycine-2-C^{14}

The fibroin was isolated from the radioactive cocoon fibres produced by the silkworms (Nichi 122×Si 115) having consumed 0.5μc per larva of glycine-2-C^{14} at the 4th day of the 5th instar. 2 g of the fibroin was hydrolyzed with 12 ml of 6 N hydrochloric acid for 15 hours. From the hydrolysate, tyrosine, glycine and alanine were isolated according to the procedure described in a previous paper[4], and the other amino acids were isolated by ion exchange column method, following the Stein and Moore's procedure[5]. The chemical purity of these isolated amino acids was confirmed by paper chromatography, and the radioactivity of these amino acids were measured by the SC-16 Windowless Gas Flow Counter (Table II).

RESULTS

The distribution of C^{14} on the silk filaments produced by the silkworms having consumed the radioactive mulberry leaves in different stages from the beginning of the 5th instar to the end is shown in Fig. 3. C^{14} appeared in abundance on the first 100 m of the silk filament at the 4th day of the 5th instar, and on the section from 200 m to 400 m at the 6th day, and on the section from 700 m to 1,000 m at the 8th day. Furthermore Fig. 3 shows that the C^{14} exists also in each silk filament at a certain extent from 700 m to 1,200 m, in common with

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[4] T. Fukuda, *J. Biochem.*, **43**, 137 (1956).

[5] C.H.W. Hirs, S. Moore and W.H. Stein, *J. Biol. Chem.*, **195**, 669 (1952).

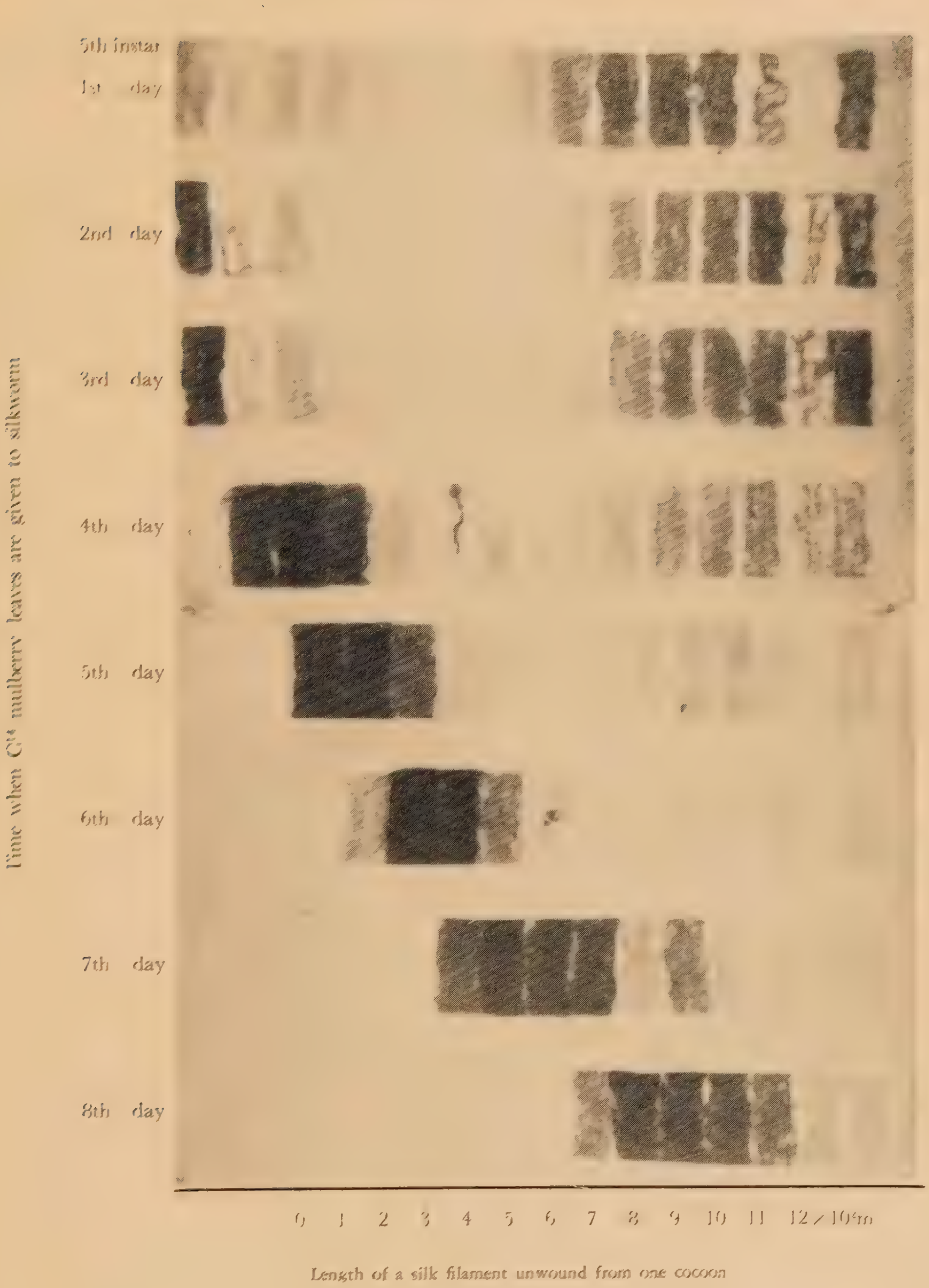

Fig. 3. Radioautograph of the silk filaments

The black part shows the existence of C^{14}

TABLE I. Radioactivity of amino acid spots on the chromatogram of the acid hydrolysates of the fibroin produced by the silkworms having consumed mulberry leaves labeled with C^{14} at the 4th day of the 5th instar

Amino acid	cpm/3cm^2
Serine	274
Alanine	39
Glycine	13
Tyrosine	4
Glutamic acid	3
Aspartic acid	1

TABLE II. Radioactivity of amino acids isolated by ion exchange column method from the fibroin produced by the silkworms having consumed glycine-2-C^{14} at the 4th day of the 5th instar

Amino acid	cpm/mg of amino acid	cpm/amino acid of fibroin 100 mg
Glycine	1,048	44,854
Alanine	24	779
Serine	27	397
Glutamic acid	8	11
Aspartic acid	4	7
Lysine	8	3
Arginine	2	2
Histidine	4	1
Leucine	1	1
Threonine	0	0
Valine	0	0
Methionine	0	0
iso-Leucine	0	0

all silkworms except the silkworm at the 8th day of the 5th instar. The same phenomenon was also obtained when C^{14} glycine, C^{14}-L-serine and C^{14}-DL-alanine were employed instead of the radioactive mulberry leaves in this experiment.

The radioactivity of each amino acid isolated from the radioactive silk produced by the silkworms having consumed the radioactive mulberry leaves and glycine-2-C^{14} at the 4th day of the 5th instar is shown in Table I and Table II. The C^{14} existed in the amino acids, such as serine, alanine, glycine, tyrosine, glutamic acid and aspartic acid in the former case, and in the amino acids such as glycine, alanine, serine, glutamic acid, aspartic acid etc. in the latter case. These facts show that the radioactivity of the silk produced by the silkworms having consumed radioactive mulberry leaves and radioactive amino acids is not due to the contamination of the surface of the silk by the C^{14}, but is due to the existence of the C^{14} in the molecules of the constituent amino acids of the silk.

DISCUSSION

In the present study, the distribution of C^{14} on the silk filaments produced by the silkworms having consumed the radioactive mulberry leaves in different stages from the beginning of the 5th instar to the end was investigated (Fig. 3). Fig. 3 shows that the first 100 m of the silk filament unwound from one cocoon is made from the mulberry leaves that the silkworm ate on the 4th day of the 5th instar: the section from 200 m to 400 m from those consumed on the 6th day of the 5th instar, and the section from 700 m to 1,000 m from those consumed on the 8th day of the 5th instar, namely just before reaching maturity. This experimental result seems to suggest that a great amount of the silk produced by one silkworm is directly derived from the mulberry leaves, and that this direct formatson of the silk takes place during the period from the 4th day of the 5th instar

TABLE III. Incorporation of glycine-1-C^{14} into the proteins of the body fluid and the proteins of the silkgland

Time elapsed	Body fluid proteins	Silkgland	
		Middle division	Posterior division
hr	cpm/mg	cpm/mg	cpm/mg
0.30	0	2	6
1.00	0	1	7
1.15	0	1	9
1.30	0	2	61
1.45	2		
2.00	2		
2.30	3		
3.00	11	9	125
3.30	15	14	91
4.00	16	14	133
6.00	21	9	56
8.00	35	23	103
10.00	80	29	111
24.00	103		

After 0.1 μc of glycine-1-C^{14} per larva was given per os to silkworms on the 6th day of the 5th instar, the body fluid and the silkglands were obtained from these silkworms at regular intervals after administration of the radioactive glycine. The proteins of the body fluid were obtained by adding the solution of trichloracetic acid several times and dried. The silkglands obtained were dried, and were divided into the middle division and the posterior division. Radioactivities of each sample were measured by the SC-16 Windowless Gas Flow Counter.

to maturity. It can also be concluded from Table III which shows that part of the glycine labeled with C^{14} given per os to the silkworm is used more quickly for the formation of the silkproteins than for the formation of the proteins in the body fluid, that the silkproteins are directly derived from the amino acids of the mulberry leaves. Furthermore, Fig. 3 shows that the C^{14} exists also in each silk filament to a certain extent from 700 m to 1,200 m in common with all silkworm except the silkworm at the 8th day of the 5th instar, namely just before reaching mrturity. This fact seems to suggest that the section from 700 m to 1,200 m of the silk filament is derived also from the mulberry leaves that silkworms ate at the beginning of the 5th instar and at the middle, i. e., the formation of this section of a silk filament is due to the temporary transportation of proteins from the larval tissues and body fluid proteins to the silkglands which takes place in relation to the metamorphosis of the silkworm larva.

In a previous paper[6], the author found that the weight of the silkglands of the silkworm ligated* tightly with a thread at the position between the head and the prothoracic segment on the 3rd day of the 4th instar, increases, even under starvation, owing to the formation of the silk substances. This fact gives strong support to our claim that some of the silkproteins are derived also from the tissue proteins in relation to the metamorphosis of the silkworm larva. The indirect formation of the silk does not take place in a mechanism known as "turnover of substances" at the larval tissues, such as alimentary canal, muscles, fatty bodies, because, in this case, the C^{14} in a silk filament appears to a certain extent from the middle to the rear. However, the author does not deny the existence of a mechanism "turnover" at the silkworm tissues and the formation of the silk based upon this mechanism. The comparatively weak C^{14} exists, as shown in Fig. 3, in all the parts of the silk filament except those produced directly from the mulberry leaves and those from the middle to the rear of a silk filament. This C^{14} might be due to the mechanism "turnover of substances" at the silkworm tissues. The occurrence of the C^{14} is not remarkable in the first 600 m of a silk filament produced by the silkworms having consumed the radioactive mulberry leaves at the 8th day of the 5th instar. The same phenomena are also recognized in the silk filaments produced by the silkworms having consumed the radioactive mulberry leaves at the 6th and 7th day of the 5th instar. These facts seem to suggest that a mechanism "turnover" between a constituent amino acid in the fibroin which has already formed and an amino acid is not active in the silkgland.

SUMMARY

1) The present study was carried out to clarify a mechanism of the formation of the silkprotein during the growth of the silkworm larva.

2) A large part, about 70 per cent of the silkproteins produced by one silkworm is directly derived from the proteins of the mulberry leaves (direct formation), but some part of the silkproteins, about 30 per cent, is also derived from the tissue proteins and body fluid proteins, in relation to the metamorphosis of the silkworm larva (indirect formation); there are two pathways in the formation of the silkproteins during the growth of the silkworm larva, i. e., direct formation and indirect formation.

3) The direct formation of the silkproteins takes place, in the hybrids of Japanese race and Chinese race, during the period between the 4th day of the 5th instar and maturity, but the indirect formation takes place during the period from the 8th day of the 5th instar to just before termination of spinning after reaching maturity.

My thanks are due to Mr. Mitsumasa Suto, Mr. Motoichi Matuda, Mr. Tsutomu Kurose and Mr. Yoshiaki Horiuchi for their skilful technical assistance in the current work.

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* This ligated larva is, under starvation, precociously matured after 96 hours and then becomes precocious pupa after 168 hours.

[6] T. Fukuda, *Bull. Sericult. Exp. Sta.* (Japan), **13**, 423 (1951).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 402~404, 1960]

Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia

Part V. Syntheses of (−)-Isopulegol and (+)-Neo-isopulegol*

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(−)-Isopulegol and (+)-neo-isopulegol were isolated by a partial esterification of isopulegol mixture, obtained by the hydrolysis of the condensation product of (+)-citronellal with acetic anhydride. From the infrared spectra and optical rotation, it has been shown that isopulegol synthesized from (+)-citronellal mainly consists of (−)-isopulegol and (+)-neo-isopulegol in approximately equal amounts, but the presence of other isomers is negligible.

Isopulegol, obtained by the hydrolysis of the condensation product of citronellal with acetic anhydride, is a mixture of stereoisomerides, since in the cyclisation of citronellal, two additional asymmetric carbon atoms are formed. Therefore, isopulegol prepared from (+)-citronellal can exist in four stereoisomerides, namely, (−)-isopulegol, (+)-neo-isopulegol, (+)-iso-isopulegol and (+)-neoiso-isopulegol.

By means of fractional crystallisation of the magnesium salt of the hydrogen phthalate, Pickard, Hunter, Lewcock and Pennington[1] succeeded in isolating two alcohols, (−)-isopulegol and (+)-neo-isopulegol which they named *d*-α-isopulegol. The former alcohol was obtained in a pure state, whilst the latter was somewhat impure.

Short and Read[2], by reduction of (+)-pulegone with aluminum-isopropoxide, have recently prepared pure alcohol in a 1.5% yield. On hydrogenation, this gave pure (+)-neomenthol, and it was concluded that this alcohol is (+)-neo-isopulegol.

However, (+)-neo-isopulegol has not been obtained in a pure state from isopulegol synthesized from (+)-citronellal, and there is much room left for investigation of the contents of isomerides.

The present study was undertaken in order to determine the composition of isomerides in isopulegol synthesized from (+)-citronellal, and to obtain the isopulegol isomers in a pure state, which is indispensable for identification of the reduction product of (+)-isopulegone with sodium in aqueous ammonia, i. e., our method of reduction employed in this series.

Isopulegol, synthesized from (+)-citronellal, was divided into three fractions, according to the partial esterification method reported by Read[3] in menthol series. Then, (−)-isopulegol and (+)-neo-isopulegol were isolated in a pure state, through the formation of hydrogen phthalate and 3,5-dinitrobenzoate respectively. Both isomers were also related to (−)-menthol and (+)-neomenthol respectively, by catalytic hydrogenation.

From the infrared spectra and the optical rotation, it was found that isopulegol, synthesized from (+)-citronellal, mainly consists of (−)-isopulegol and (+)-neo-isopulegol in approximately equal amounts, and other isomerides were negligible. This fact was proved from the optical rotations of menthyl acetate mixture and menthol mixture, prepared by the catalytic hydrogenations of isopulegyl acetate and isopule-

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* For Part IV see This Bulletin, **24**, 90 (1960).

** Laboratory of Agricultural Chemistry, Faculty of Agriculture, Shinshu University.

[1] R.H. Pickard, H. Hunter, W. Lewcock and H.S. Pennington, *J. Chem. Soc.*, **1920**, 1248.

[2] A.G. Short and J.H. Read, *J. Chem. Soc.*, **1939**, 1306; cf. A.K. Macbeth and J.S. Shannon, *J. Chem. Soc.*, **1952**, 4748.

[3] W.J. Grubb and J. Read, *J. Soc. Chem. Ind.*, **53**, 53T (1934).

TABLE I. ISOPULEGOL FRACTION PREPARED BY THE PARTIAL ESTERIFICATION

	Isopulegol–I	Isopulegol–II	Isopulegol–III
Yield (g)	7.4	7.2	10.3
b.p./mmHg.	71.5∼72°/5mm	69∼70°/6mm	62∼62.5°/4mm
d_4^{24}	0.9062	0.9074	0.9093
n_D^{24}	1.4688	1.4692	1.4659
MR	47.39	47.36	46.97
α_D^{32}	−12.0°	−10.6°	+16.0°

gol, respectively.

A small amount of 3,5-dinitrobenzoate, $C_{17}H_{20}O_6N_2$ (one double bond), m. p. 152∼153°C, was also isolated, but its structure was not confirmed as yet.

EXPERIMENTAL

All melting and boiling points were uncorrected. Microanalyses were carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto. Infrared spectra were recorded with a Shimadzu double beam spectrophotometer at Prof. Nakajima's Laboratory, University of Kyoto.

Preparation of Isopulegol from (+)-Citronellal

Isopulegyl acetate was prepared by the treatment of (+)-citronellal (159 g) with acetic anhydride (340 g), according to the procedure of Tiemann and Schmidt[4]; Yield 127 g (62.9%), b. p. 83∼87°/6 mm, d_4^{26}0.9329, n_D^{26} 1.4533, MR 56.89 (Calcd. 56.60), $[\alpha]_D^{26}-0.9°$ (homog.), E. V. 275.85 (Calcd. 285.82). On hydrogenation at 33° under 80 Kg/sq. cm pressure maximum with Raney-Ni, it gave menthyl acetate mixture, b. p. 92.5∼95°/8.5 mm, n_D^{30} 1.4421, $\alpha_D^{27}-23.84°$ (homog.).

Isopulegol was obtained by hydrolysis of the above isopulegyl acetate (116 g); Yield 84 g (92.0%), b. p. 80.5∼81.5°/9mm, d_4^{26} 0.9054, n_D^{26} 1.4675, MR 47.31 (Calcd. 47.24), $[\alpha]_D^{26}+0.4°$ (homog.). When isopulegol was hydrogenated with platinum oxide in acetic acid, menthol mixture was obtained; b. p. 94.5∼96.5°/9 mm., d_4^{29} 0.8946, $[\alpha]_D^{29}-15.43°$ (homog.).

Fractional Esterification of Isopulegol

To a solution of isopulegol (30.0 g) in pyridine (50 ml) was added slowly one-third of the calculated amount (12.0 g) of *p*-nitrobenzoylchloride, the mixture being cooled with water at 24∼26° and stirred during the addition. After further stirring (40 mins.), the product was treated as usual method. Steam distillation of the product gave ester (Ester-I) (16.9 g), d_4^{34} 1.1126, n_D^{34} 1.5300, $[\alpha]_D^{34}-31.3°$ (homog.), and recovered unesterified isopulegol (19.7 g), b.p. 68.5∼69.0°/4 mm, d_4^{29} 0.9034, n_D^{29} 1.4663, MR 47.31, $[\alpha]_D^{29}+5.1°$ (homog.). The unesterified isopulegol (19.5 g) was esterified again with half of the calculated amount (12.0 g) of *p*-nitrobenzoylchloride according to the above mentioned method, to give Ester-II (17.2 g), d_4^{34} 1.1034, n_D^{34} 1.5260, $[\alpha]_D^{34}-26.3°$ (homog.), and unesterified isopulegol (Isopulegol-III). Ester-I and Ester-II were hydrolysed under reflux in potassium-hydroxide alcoholic solution and gave Isopulegol-I and Isopulegol-II, respectively. Isopulegol-I, II and III possessed the properties as shown in Table I. No difference was observed between Isopulegol-I and Isopulegol-II.

(−)-Isopulegyl Hydrogen Phthalate

Isopulegol-II (6.95 g) was heated at 110° for 17 hrs. with phthalic anhydride (8.0 g). The product was worked up in the usual manner and gave crude isopulegyl hydrogen phthalate (11.5 g) as a viscous yellow oil. The phthalate was dissolved in a cold aqueous solution of the calculated amount of sodium carbonate (500 ml), and the solution was preciptated with a calculated amount of solution of magnesium chloride. The resulting flaky crystal (10.77 g), m. p. 101∼104.5°, was recrystallized from water containing methanol. Isopulegyl phthalate magnesium salt (6.36 g, Yield 38.4% from Isopulegol-II) obtained after two crystallizations had m. p. 108∼109°, $[\alpha]_D^{30}+5.0°$ (c, 27.948 in methanol), *Anal.* Found: C, 59.19; H, 7.34. Calcd. for $C_{36}H_{42}O_8Mg\cdot 6H_2O$: C, 58.82; H, 7.40%. On the other hand, from Isopulegol-I (7.12 g), magnesium salt (7. 75g, Yield 45.7% from Isopulegol-I), m. p. 104∼105°, $[\alpha]_D^{30}+5.1°$ (c, 27.324 in methanol) was obtained, according to the same method as described above. From the optical rotation, both magnesium salts probably contained a considerable amount of (+)-neo-isopulegyl phthalate-Mg-salt. However, they could not be purified satisfactorily by recrystallization.

Thereafter, the combined magnesium salt (14.01 g) was decomposed by a cold hydrochloric acid and gave crude isopulegyl hydrogen phthalate (11.27 g, Yield 97.1%), m. p. 77.5∼83.5°. Two crystallizations of crude phthalate from *n*-hexane gave pure (−)-isopulegyl hydrogen phthalate (6.24 g, 55.4% recovery from crude phthalate), m. p. 101~102°, not altered on further recrystallizations from *n*-hexane or acetic acid-water; $[\alpha]_D^{30}-21.0°$ (c, 40.00 in methanol), *Anal.* Found: C, 71.66; H, 7.48, Calcd. for $C_{18}H_{22}O_4$: C, 71.50; H, 7.33%.

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[4] F. Tiemann and R. Schmidt, *Ber.*, **30**, 22 (1897).

(−)-Isopulegol

(−)-Isopulegyl hydrogen phthalate (4.95 g) was hydrolysed under reflux for one hour in ethanol (50 ml) containing potassium hydroxide (2.3 g). (−)-Isopulegol (2.12 g, Yield 84.1%) was obtained by distillation as a colorless oil with a mild menthol-like odor; b. p. 74.0°/6.5 mm., d_4^{26} 0.9062, n_D^{26} 1.4690, MR 47.40 (Calcd. 47.24), $[\alpha]_D^{26}-23.6°$ (homog.).

Esterification of (−)-isopulegol (237 mg) with 3,5-dinitrobenzoylchloride in pyridine gave (−)-isopulegyl 3,5-dinitrobenzoate (514 mg, Yield 96.1%), m. p. 104~104.5°, *Anal.* Found: C, 58.89; H, 5.92. Calcd. for $C_{17}H_{20}O_6N_2$: C, 58.61; H, 5.79%.

(−)-Isopulegol (1.005 g) was dissolved in acetic acid (7 ml) and hydrogenated by using platinum oxide (20 mg) as a catalyst. It absorbed 164 ml (26°) of hydrogen and gave (−)-menthol (0.803 g, Yield 78.8%), b. p. 78.0°/6 mm, m. p. 41.7~42.5°, undepressed by admixture with an authentic sample (m. p. 42.2°).

(+)-Neo-isopulegyl 3,5-Dinitrobenzoate

Isopulegol-III (9.81 g) was esterified with 3,5-dintrobenzoylchloride (20.0 g) in pyridine (40 ml). By steam distillation of the product, a crude ester (18.55 g, Yield 83.7%) was isolated from unesterified oil (see belows). Two crystallizations of the crude ester from *n*-hexane gave (+)-neo-isopulegyl 3,5-dinitrobenzoate, as pale yellow needles, (9.58 g, Yield 43.2% from Isopulegol-III), m. p. 133.0~133.5°, and it remained constant after further recrystallizations; *Anal.* Found: C, 58.62; H, 5.58. Calcd. for $C_{17}H_{20}O_6N_2$: C, 58.61; H, 5.79%.

The unesterified oil (1.148 g), from the above steam distillation, was completely converted into 3,5-dinitrobenzoate (0.339 g). After crystallizations twice from *n*-hexane, 3,5-dinitrobenzoate (0.129 g), m. p. 152~153°, was obtained; *Anal.* Found: C, 58.78; H, 6.01. Calcd. for $C_{17}H_{20}O_6N_2$: C, 58.61; H, 5.79%. The infrared spectrum of this substance did not agree with the spectra of 3,5-dinitrobenzoate of (−)-isopulegol and (+)-neo-isopulegol.

(+)-Neo-isopulegol

(+)-Neo-isopulegyl 3,5-dinitrobenzoate (6.01 g) was hydrolysed under reflux for one hour in ethanol (50 ml) containing potassium hydroxide (1.2 g). (+)-Neo-isopulegol (2.31 g, Yield 86.7%) was a colorless oil possessing an odor very similar to that of (+)-neomenthol; b. p. 70.5°/6.5 mm, d_4^{26} 0.9121, n_D^{26} 1.4708, MR 47.25 (Calcd. 47.24), $[\alpha]_D^{26}+34.4°$ (homog.).

Esterification of (+)-neo-isopulegol (232 mg) with *p*-nitrobenzoylchloride in pyridine gave (+)-neo-isopulegyl *p*-nitorobenzoate (443 mg, Yield 96.9%), m. p. 91.5~92.2°.

(+)-Neo-isopulegol (1.006 g) readily underwent hydrogenation in acetic acid (7 ml) in the presence of a platinum oxide (20 mg). The hydrogenation product was a colorless oil; b. p. 82.2~82.5°/8 mm, d_4^{22} 0.8984, n_D^{22} 1.4604, MR 47.67 (Calcd. 47.71), $[\alpha]_D^{22}+19.95°$ (homog.). These constants are closely similar to those of (+)-neomenthol. When esterified in pyridine with 3,5-dinitrobenzoylchloride, the hydrogenation product (204 mg) gave (+)-neomenthyl 3,5-dinitrobenzoate (420 mg, Yield 91.4%), m. p. 154.5~155.0°, undepressed by admixture with an authentic sample (m. p. 154°).

Infrared Spectra of Isopulegol Isomers

The infrared spectra of (−)-isopulegol and (+)-neo-isopulegol showed a strong absorption band at 889 cm^{-1} and its C=C stretching frequency appeared at 1642 cm^{-1}, which closely agreed with 889, 1641 cm^{-1} of isopulegol, reported by Macbeth⁵⁾.

From optical rotation, isopulegol synthesized from (+)-citronellal seemed to be composed of about 58% of (−)-isopulegol and 42% of (+)-neo-isopulegol. The infrared spectrum of isopulegol synthesized from (+)-citronellal closely agreed with the spectrum of a mixed sample prepared by direct weighing of (−)-isopulegol (120.1 mg) and (+)-neo-isopulegol (85.5 mg), in the same proportion as mentioned above. Any absorption band attributable to the presence of other isomerides was not shown in this spectrum.

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5) A.K. Macbeth and J.S. Shannon, *J. Chem. Soc.*, **1952**, 4748.

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Whiteness of Cigarette Ash

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Effects of physical and chemical properties on whiteness of cigarette ash have been studied. The whiteness of cigarette ash could be measured successively with a Photovolt Photoelectric Reflection Meter. In cigarettes made from Bright leaves, the reflectance has close relationships with weight per cigarette and the smoking conditions such as puff volume and frequency of puff; on the contrary, in cigarettes made from Burley or Japanese Native leaves, the reflectance is almost constant under any weight per a cigarette and any smoking conditions. The whiteness of cigarette ash of various tobacco leaves decreases in the following order: Bright (and Turkish), Japanese Native "Matsukawa-ha", Burley, and Japanese Native "Daruma-ha". Both the kinds and amounts of each inorganic constituent contained in the leaves vary the whiteness of cigarette ash.

The whiteness of cigarette ash plays an important role to the burning quality of cigarettes, and it is considered to be related with the degree of carbonization of organic materials, the combustion-zone temperature of cigarettes, and other complicated "combustion phenomena" of cigarettes.

The whiteness of cigar ash has been investigated by means of visual comparison[1,2]. However, in this method, many measuring errors arise largely due to the differences of illuminations, extent of eye-fatigue, and subjectivity of an individual observer. Pyriki et al.[3] measured the whiteness of cigarette ash, using a Ostwald book of color. Although this procedure is simple and rapid, it has disadvantages due to the differences of individual subjectivity and lack of consistent numerical evaluations of whiteness. Therefore, it became essential that a rapid and objective method for evaluating the general appearance of tobacco ash should be investigated. The utilization of photoelectric instruments has already been reported in measuring the brightness (or the whiteness) of starch[4~6], sugar[7], salt[8,9], and clay[10,11]. These methods, however, can not be available directly to measure the whiteness of cigarette ash, because some differences in physical properties are observed between tobacco ash and the materials abovementioned.

A work, utilizing a Photovolt Photoelectric Reflection Meter (PPRM) has shown that good correlation exists between the visual appearance of cigarette ash and its relative reflectance to magnesium oxide[12].

Cigarette ash was prepared by artificial smoking of some different cigarettes respectively

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[1] J. Johnson, *Agr. Exp. Sta. of Univ. Wisconsin Research Bull.*, No. **140** (1941).

[2] I. Kanai, *J. Agr. Chem. Soc. Japan*, **32**, 566 (1958).

[3] C. Pyriki and W. Philipp, *Ber. Inst. Tabakforsch., Dresden*, **2, No. 1**, 94 (1955).

[4] S. Suzuki and Y. Nemoto, *Dempun Kôgyô Gakkaishi* (*J. Technol. Soc. Starch*), **2, No. 2**, 7 (1954).

[5] S. Suzuki, T. Hirohata and Y. Nemoto, *ibid.*, **2, No. 3**, 16 (1955).

[6] S. Suzuki and T. Hirohata, *ibid.*, **3, No. 2**, 22 (1955).

[7] J.C. Keane and B.A. Brice, *Ind. Eng. Chem. Anal. Ed.*, **9**, 258 (1937).

[8] T. Harada and T. Masuzawa, *Bull. Soc. Salt Sci. Japan*, **10**, 234 (1956).

[9] T. Harada and T. Masuzawa, *ibid.*, **11**, 268 (1957).

[10] R.J. Casey, "Lectures on Pulp and Paper Manufacture (Univ. Maine)", **Series II**, 222 (1953).

[11] Tappi, T 646 m (1958), Tech. Assoc. Pulp Paper Ind., New York.

[12] I. Kanai, Unpublished Data.

made from a single variety of tobacco. After drying, the ash was ground in a mortar and screened through a sieve. Then, the ash was poured into a metal dish, and the top surface of the ash layer was smoothed with a cylindrical metal piece. The reflectance of ash sample thus prepared was measured by a PPRM, and the whiteness of cigarette ash was experessed in terms of the percentage reflectance to a standard plaque of the PPRM.

Attempts were conducted to investigate the relationship between the whiteness of cigarette ash and smoking conditions, moisture contents of cigarettes, weight per cigarette, or varieties of leaf tobaccos. It was found that these relationships were different in Bright, Japanese Native and Burley tobaccos, respectively. Furthermore, it was indicated that inorganic constituents contained in leaf tobaccos affected the whiteness of cigarette ash.

EXPERIMENTAL

Variety of Leaf Tobacco

Kinds of the leaves used for sample cigarettes were the following ten; Japanese Bright ("cutter" and "leaf"), Japanese Native "Matsukawa-ha" ("cutter" and "leaf"), Japanese Native "Daruma-ha" ("cutter" and "leaf"), Japanese Burley ("cutter" and "leaf"), U.S. Bright (MBL), and Turkish (Izmir-B).

Cigarette Paper

Cigarette paper employed in this experiment was made of hemp, flax or ramie, and did not contain any wood fibers.

Preparation of Sample Cigarettes and Cigarette Ash

Cigarettes were made from cut-leaves of 0.6 mm width and were 70 mm in length. These cigarettes were smoked by an artificial smoking device[13] and ashes were collected from the smoked portion of 3 cm between 1 and 4 cm from lighted end. After drying for one hour at 100°C, the ashes were grounded in a mortar, screened through a 0.125 mm-sieve, and stored over calcium chloride in a desiccator.

Instrument and Procedure of Reflectance Measurements

A Photovolt Photoelectric Reflection Meter (PPRM) was employed for measuring the whiteness of cigarette ash. The filter attached is a green tristimulus one. A working standard used in this experiment is a steel plaque coated with porcelain enamel, having a reflectance of 75% to freshly prepared magnesium oxide surface. A black hollow-cylindrical cavity is 50 mm in depth and 33 mm in inside diameter. An iron cuvette, plated with brass, is cylindrical, 33 mm in depth, and 22 mm in inside diameter.

The cigarette ash is poured into the cuvette mentioned above and the top surface of the ash layer is smoothed once or twice by rotating a 100 g weight without any hand pressure. The smoothing action is repeated until the surface of the ash becomes accurately flush with the edge of the cuvette. Then, the whiteness of the ash is measured by the PPRM attached the green filter and is expressed in terms of percentage reflectance to the standard plaque.

RESULTS AND DISCUSSION

1. Effect of Smoking Conditions

Puff volume: Mean weights per a sample cigarette were 945±4.0 mg in Japanese Bright ("leaf"), 617±3.0 mg in "Matsukawa-ha" ("leaf"), and 617±3.7 mg in Japanese Burley ("leaf") at 0.05 confidence level. Moisture contents of cigarettes were 14.4% in Bright, 11.8% in "Matsukawa-ha", and 12.0% in Burley. The measurements of reflectance were carried out on the ashes obtained by free burning, continuous- (puff volume—40 ml/2sec) and intermittent-smoking (puff duration—2 sec; frequency of puff—once/30 sec) in a room conditioned at 20°C and 60% R. H..

Fig. 1 gives the effect of puff volume on the

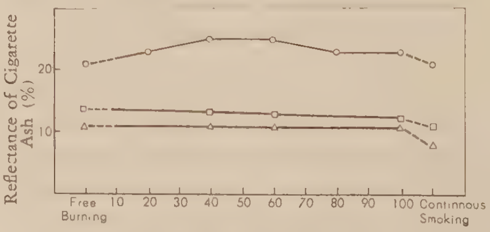

FIG. 1. Relationship between Puff Volume and Reflectance of Cigarette Ash.

—○— Japanese Bright ("leaf")
—□— "Matsukawa-ha" ("leaf")
—△— Burley ("leaf")

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[13] M. Izawa and Y. Kobashi, *J. Agr. Chem. Soc. Japan*, **29**, 754 (1955).

reflectance of cigarette ash. It was observed that the ash of Bright cigarettes was not viscous than that of "Matsukawa-ha" or Burley.

The reflectances of Bright ash were the highest* at 40 to 60 ml/puff and lower at the other puff volumes. The lowest reflectances were measured at the free burning and continuous smoking.

In Bright cigarettes, increase of the ash reflectance between the free burning and 60 ml puff volume is explained by increase of volume of air (or oxygen) supplied to combustion zone of cigarettes. At above 60 ml puff volume and continuous smoking, the sample cigarettes burned very irregularly and gave long cone-shaped burning ends. Then, suctioned air does not flow through the entire area of cone-shaped combustion zone but directly enter from the bottom of ashed cone. Consequently, cut tobaccos inside of the cone burn incompletely and give darker ashes.

On the other hand, the whiteness of "Matsukawa-ha" and Burley ashes was almost constant in the whole range of puff volumes with the exception of continuous smoking. In these cigarettes, the degree of air supply seems to be unimportant, and this fact may be explained by the more viscous or coherent ashes which are apt to enclose unburnt carbon particles.

Frequency of puff: Mean weights and moisture contents per a cigarette used in this test were 947±5.0mg, 14.4% in Japanese Bright ("leaf"), 619±3.6 mg, 11.8% in "Matsukawa-ha" ("leaf"), and 620±2.6 mg, 12.0% in Burley ("leaf"), respectively. The ranges of the mean weights per a cigarette were all indicated at 0.05 confidence limit.

In Bright tobaccos, the reflectance of ash varied with the frequency of puff (the maximum value was observed at one puff per 10 sec); while, in "Matsukawa-ha" and Burley it gave nearly constant values with the exception of continuous smoking (Fig. 2).

It may be explained by volume of air supplied to combustion zone and irregular burning of cigarettes that there was a difference of the effect of puff-frequency on the reflectance of cigarette ash between Bright and a "Matsukawa-ha"—Burley group, as mentioned in "the relationship between puff volume and the reflectance of cigarette ash." It has been reported that average puff volume and puff-frequency of human smokers are 40 ml/puff and once per 30 sec, respectively[14]. The author's experiment showed these standard smoking conditions gave the ashes of the highest reflectance. Consequently, it is concluded that the actual human smoking conditions are favorable for comfortable smoking because of white and fine cigarette ashes.

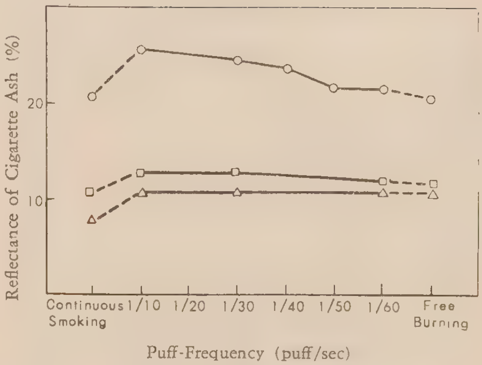

FIG. 2. Relationship between Puff-Frequency and Reflectance of Cigarette Ash.

—○— Japanese Bright ("leaf")
—□— "Matsukawa-ha" ("leaf")
—△— Burley ("leaf")

2. Effect of Moisture Content of Cigarette

The mean weight of cigarettes, stored in a room conditioned at 20°C and 60% R. H., were 1,100±10 mg in Bright ("leaf") tobaccos, and 700±10 mg in both "Matsukawa-ha" ("leaf") and Burley ("leaf") tobaccos. The ranges of the mean weight are indicated at 0.05 confidence level. The sample cigarettes thus stored were re-humidified under the five conditions (40, 50, 60, 70, and 80% R. H. at 25°C) over sulfuric acid in desiccators. The ashes of 25 to 30 cigarettes were obtained by intermittent smoking

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* Higher reflectance means whiter ashes.

[14] B. Pfyl, *Z. Untersuch. Lebensm.*, **66**, 501 (1933).

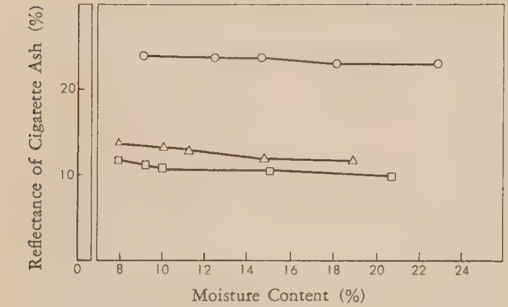

FIG. 3. Relationship between Moisture Content and Reflectance of Cigarette Ash.

—○— Japanese Bright ("leaf")"
—△— "Matsukawa-ha" ("leaf")
—□— Burley ("leaf")

(puff volume—20 ml/sec; puff duration—2 sec; frequency of puff—once/30 sec).

Fig. 3 gives the effect of the moisture content on the reflectance of cigarette ash. The cigarettes of more moisture contents seem to give whiter ashes, but generally, it is not too much to say that the moisture contents of cigarettes inconsiderably affect the reflectance of ash between 40 to 80% R. H. In the range of 40 to 80% R.H. at 25°C, the moisture contents of Bright tobaccos were about 9 to 23% and those of either "Matsukawa-ha" or Burley tobaccos were about 8 to 19 (or 20)%. From the results obtained, it is found that the moisture contents of cigarettes are not an important factor to the whiteness of cigarette ash.

3. Effects of Varieties of Leaf Tobaccos and Weight per a Cigarette

The moisture contents of sample cigarettes were as follows; 14.6 to 14.7% in Japanese Bright, 10.9 to 11.2% in "Matsukawa-ha", 9.7% in "Daruma-ha", 9.7 to 9.8% in Japanese Burley, 12.4% in U. S. Bright, and 10.0% in Turkish tobaccos. The numbers of sample cigarettes were different, 10 to 80 cigarettes, as it was very difficult to prepare cigarettes having uniform weights. The ash was obtained by intermittent smoking (puff volume—20 ml/sec; puff duration—2 sec; frequency of puff—once per 30 sec).

Results obtained are given in Fig. 4 and 5.

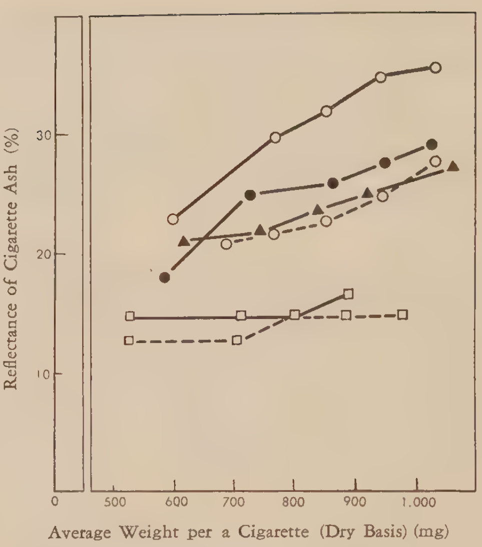

FIG. 4. Effects of Varieties of Leaf Tobaccos and Weight per a Cigarette on Reflectance of Cigarette Ash. (1).

—○— Japanese Bright ("cutter")
---○--- Japanese Bright ("leaf")
—●— U.S. Bright (MBL)
—▲— Turkish (Izmir-B)
—□— "Matsukawa-ha" ("cutter")
---□--- "Matsukawa-ha" ("leaf")

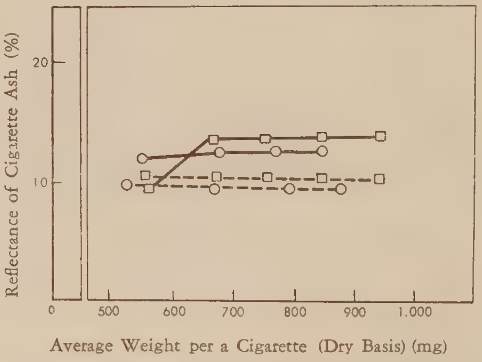

FIG. 5. Effects of Varieties of Leaf Tobaccos and Weight per a Cigarette on Reflectance of Cigarette Ash. (2).

—○— "Daruma-ha" ("cutter")
---○--- "Daruma-ea" ("leaf")
—□— Burley ("cutter")
---□--- Burley ("leaf")

The reflectances of cigarettes formed a series of Bright, "Matsukawa-ha", Burley, and "Daruma-ha" (high→low). The remarkable

TABLE I. COMPOSITION OF INORGANIC CONSTITUENTS IN TOBACCO LEAVES AND REFLECTANCES OF CIGARETTE ASHES

No.	Sample Variety	Chemical Composition (%, dry basis)							Reflectance of Ash	
		Cl	K_2O	CaO	MgO	Tot. Ash	Sol. Ash	The Other Inorganic Constituents*	(a)**	(b)***
1	Japanese Bright ("cutter")	0.28	3.20	2.24	0.40	12.22	11.28	5.16	35	35
2	Japanese Bright ("leaf")	0.70	2.72	2.17	0.39	10.97	10.34	4.36	25	25
3	"Matsukawa-ha" ("cutter")	1.29	7.57	3.26	0.58	21.92	21.50	8.80	15	15
4	"Matsukawa-ha" ("leaf")	1.22	7.38	3.40	0.62	21.66	21.36	8.74	13	13
5	"Daruma-ha" ("cutter")	1.11	9.76	2.81	0.53	23.80	22.28	8.07	12	13
6	"Daruma-ha" ("leaf")	0.91	9.19	2.41	0.54	21.50	21.06	8.01	10	10
7	Japanese Burley ("cutter")	2.03	9.31	3.72	0.74	23.75	22.95	7.15	12	13
8	Japanese Burley ("leaf")	2.26	8.03	3.40	0.75	21.88	21.54	7.10	11	11
9	U.S. Bright (MBL)	1.00	2.97	1.78	0.52	9.70	9.20	2.93	28	28
10	Turkish (Izmir-B)	1.36	2.97	3.45	1.29	21.52	15.47	6.42	26	26

Remarks: (1) * "The other inorganic constituents" = Sol. ash − ($Cl+K_2O+CaO+MgO$).

** Average weights (dry basis) per a cigarette are 600 mg in Japanese Native——Burley group, and 950 mg in Bright——Turkish group.

*** Average weights (dry basis) per a cigarette are 700 mg in Japanese Native——Burley group, and 950 mg in Bright——Turkish group.

(2) Procedures of quantitave analysis are volumetric for chlorine[15] and calcium[16], flame photometric for potassium[17], and the official analysis methods of Japan Monop. Corp. for magnesium, total ash and soluble ash[18].

differences of the reflectances could not be recognized among Japanese Bright, U. S. Bright, and Turkish tobaccos. However, there was a considerable difference of the reflectances between a Bright—Turkish tobacco group and a Japanese Native—Burley one. Furthermore, the leaf tobaccos of "cutter" position gave whiter ashes than those of "leaf" position in all varities.

In Bright and Turkish tobaccos, the reflectance of the ash increased with the weight of a cigarette; while, in Japanese Native and Burley tobaccos, it was almost constant.

Papers concerning the relationship between "the whiteness of ash" and the varieties of leaves have never been found, with the exception of the report that the ashes of Turkish tobacco are whiter than those of German tobacco[3]. The effect of varieties of leaf tobaccos on the whiteness can not be explained only by the physical factors, such as burning velocity, pressure drop or combustion-zone tempeature of cigarette. The reflectance of cigarette ash may also be affected by chemical factors mentioned below.

4. Effect of Inorganic Constituents

Inorganic constituents contained in tobacco leaves; Table I shows the percentage of chlorine, potassium, calcium, magnesium, total ash, solbule ash, "the other inorganic constituents" contained in the leaves, and the reflectance of cigarette ash.

From the Table, it is evident that the amounts of chemical constituents in tobacco leaves exert a pronounced effect on the reflectance of cigarette ash. For example, sample No. 8 contained relatively high amounts of potassium and chlorine and gave the lowest reflectance. On the other hand, sample No. 1 contained small amounts of potassium and chlorine and gave the highest reflectance. A careful examination of the present data suggest that the reflectance of ash is influenced by all of the constituents contained in the leaves. However, it can not

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[15] Association of Official Agricultural Chemists, "Official Methods of Analysis of the A.O.A.C.", **1955**, 116.

[16] S. Kubo and C. Tsutsumi, *Shokuryô Kenkyûjo Kenkyû Hôkoku (Rept. Food Research Inst.-Tokyo)*, **No. 5**, 171 (1951).

[17] T. Takahashi and D. Yoshida, *Special Bull. Hatano Tob. Expt. Sta., Japan Monop. Corp.*, **No. 2** (1954).

[18] Japan Monopoly Corporation, "Official Analysis Methods of Tobacco Leaves", 1952.

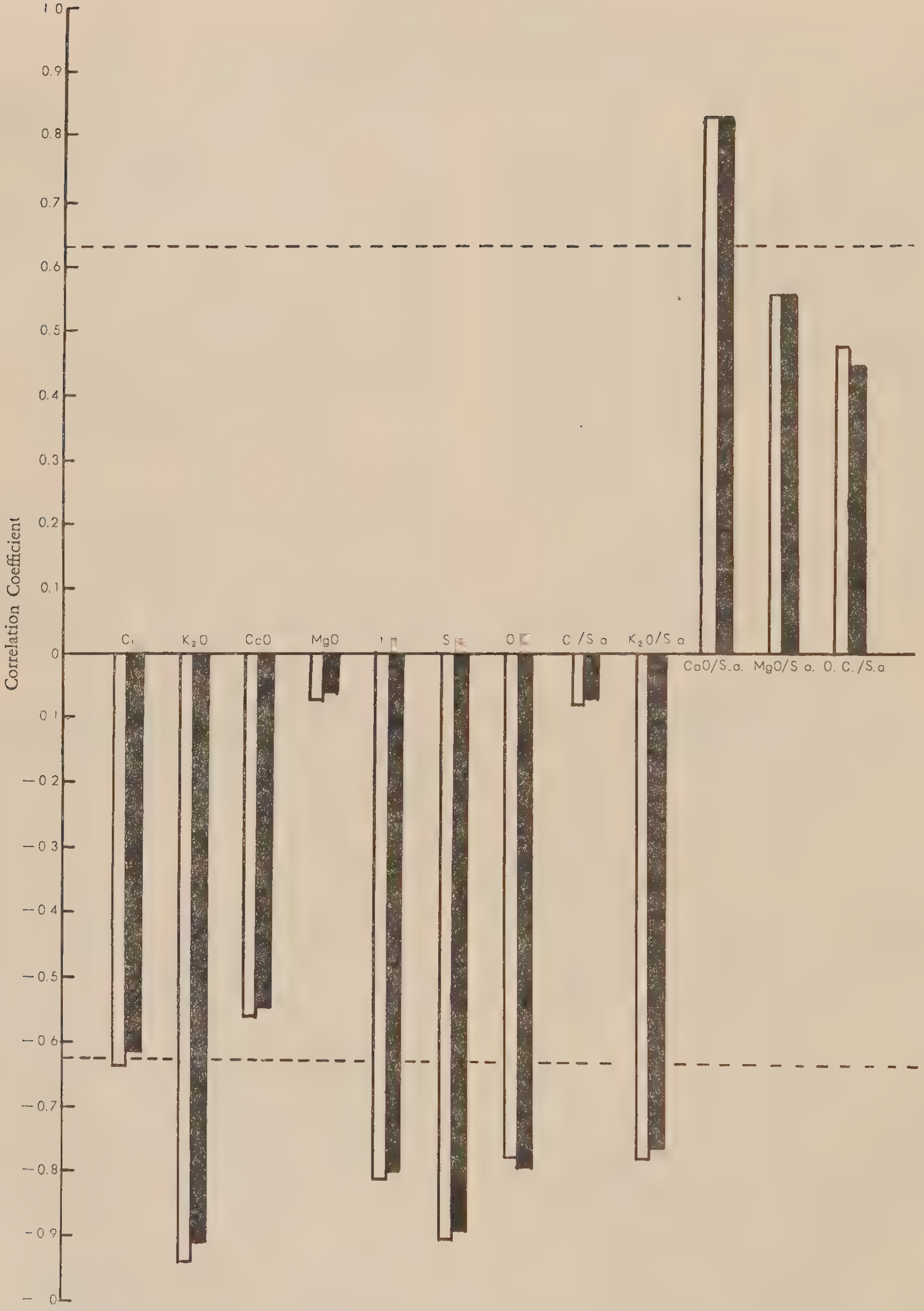

FIG. 6. Correlation Coefficients between Contents of Inorganic Constituents in Tobacco Leaves and Reflectances of Cigarette Ashes.

Remarks: (1) t.a.=total ash; s.a.=soluble ash; o.c.="the other inorganic constituents."

(2) □ ; values when average weights per a cigarette (dry basis) are 600mg in Japanese Native——Burley group, and 950mg in Bright——Turkish group. ■ ; values when average weights are 700mg in Japanese Native——Burley group, and 950mg in Bright——Turkish group.

(3) ----; significant line at 0.05 confidence level.

be found that what kind of components concerns the whiteness of cigarette ash. This may be due to the fact that the beneficial effect of some components is eliminated by reverse effect of the other components. Consequently, in order to determine the relation between each of the inorganic elements and the reflectance of ash, correlation coefficients were calculated and investigated (Fig. 6).

In this Figure, it is shown that potassium, chlorine, total ash, soluble ash, and "the other inorganic constituents" are all negative factors to the whiteness of cigarette ash, but the ratio of calcium and soluble ash is a positive factor. However, it is also considered that every constituents contained in tobacco leaves positively or negatively affect each other to the whiteness of cigarette ash. Therefore, the following spray tests of some individual inorganic salts were carried out.

Inorganic salts added to tobacco leaves; Inorganic salts, such as potassium acetate, calcium acetate, magnesium acetate, potassium chloride, calcium chloride, and magnesium chloride (2/10, 4/10, 6/10, and 8/10 mol) were dissolved in 150 ml of water, respectively. In case of calcium acetate, 4/10 mol was dissolved in 260 ml of water, and 8/10 mol—test could not be conducted owing to its low solubility. Entire volume of each solution was sprayed on 1 kg of tobacco leaves (dry basis), respectively. Sample cigarettes were made from the leaves conditioned at 20°C and 60% R. H.. Thirty cigarettes of each section, having mean weight of 1,200±20 mg per cigarette, were intermittently smoked (puff volume—40 ml/puff; puff duration—2 sec; frequency of puff—once per 30 sec) and cigarrette ashes were collected. The measurements of reflectance were carried out by the same procedure as mentioned above.

The effect of added inorganic salts on the reflectance of cigarette ash is shown in Fig. 7.

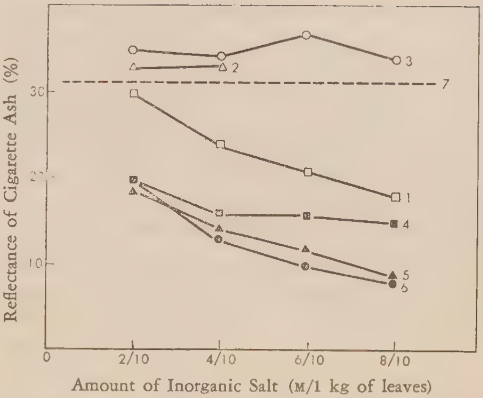

FIG. 7. Effects of Sprayed Inorganic Salts on Reflectance of Cigarette Ash.

1. Potassium arcetate 2. Calcium acetate
3. Magnesium acetate 4. Potassium chloride
5. Calcium chloride 6. Magnesium chloride
7. No Spraying

In view of the curves of acetates, magnesium and calcium are positive factors to the whiteness, but potassium seems to be otherwise. Amounts of added magnesium or calcium acetates do not give significant influence to the whiteness of cigarette ash in the range from 2/10 to 8/10 mol. In case of potassium acetate, however, addition of 2/10 mol of the salt gives the cigarette ash having similar whiteness to that of unsprayed cigarettes, and larger amounts of this salt lower the whiteness.

From the curves of chlorides, it is observed that the more amount of calcium or magnesium chloride is added to the leaves, the lower the reflectance becomes. Such lowering does not occur in the acetates with the exception of potassium acetate.

Comparison between the curves of acetates and those of chlorides evidently showed that chlorine is a detrimental factor to the whiteness of cigarette ash.

A reverse order was observed in the effect of cations between the acetates and chlorides. This may be explained by stronger negative effects of chlorine than positive effects of cations used.

Under the combustion zone temperatures of cigarettes, acetyl radical is decomposed easily to carbon dioxide and water, which are considered to be harmless to complete burning of cigarettes. Freed and combined cations with other atoms or radicals seem to be favorable to the cigarette burning and whiteness of cigarette ash.

On the other hand, the detrimental effects of chlorides may be due to the phenomena that chloride molecules do not decompose even under high temperatures of burning cigarettes and extremely unfavorable effects of chlorine eliminate the favorable effects of cations such as magnesium and calcium.

It has been reported by several authors that potassium is a beneficial element to burning velocity or burning durability of "tobacco leaves." Consequently, researches on the relationship between potassium and "burning of cigarette" or "whiteness of cigarette ash" must be made in detail by many workers.

Acknowledgements The author expresses his thanks to Director S. Nakamura, Dr. I. Ōnishi and Mr. M. Izawa of this Research Institute for support and encouragement of this work. He is also grateful to Miss Y. Baba for helping his experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 412~417, 1960]

Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part III. Adsorption of Esters of *p*-Hydroxybenzoic Acid on Yeast Cell and their Toxic Effect

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Received October 20, 1959

Alkyl esters of *p*-hydroxybenzoic acid rapidly transfer from the medium to the yeast cell, and the transferred ester is accumulated in the cell. The transfer ratio of the ester increases as the alkyl radical becomes higher, and also, its toxic effect on the yeast increases in the same manner.

In this case, a part of the transferred ester is adsorbed on the solid phase of the cell and the other part is dissolved in the lipid phase and in the inner cell fluid. When the alkyl radical of the ester is changed, it is observed that a definite quantity of the adsorbed ester is required in order to give a definite inhibiting effect on the yeast growth, though the concentration of the dissolved ester in the lipid phase and in the inner cell fluid changes. Accordingly, it is concluded that the toxic effect mainly depends upon the adsorbed esters, being independent of the dissolved ester in the lipid phase or in the inner cell fluid.

Hitherto it has been well known that the toxic effect of the alkyl ester of *p*-hydroxybenzoic acid on microbes increases as the alkyl radical becomes higher[1~5]. In connection with this, Hirai[5] has assumed that the hydrophyl-lipophyl balance in the molecule of the ester has a relation to the toxic effect.

In the previous report[6], it has been shown that lipophyl molecules of undissociated acid antiseptics are adsorbed on the yeast cell and the quantity adsorbed is the limiting factor of the toxic effect. In this case, since the lipid

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[1] T. Sabalitschka, *Pharm. Monateshefte*, **5**, 235 (1924).

[2] K. Kurono, N. Iwashita and S. Toriyama, *Report of Govermental Institute of Brewing, Japan*, **117**, 1 (1933).

[3] K. Kurono and N. Iwashita, *ibid*., **119**, 37 (1934).

[4] M. Ishio and K. Endo, *Report of Hygenic Laboratory, Japan*, **45**, 171 (1934).

[5] K. Hirai, *J. Pharm. Soc. Japan*, **77**, 1279 (1957).

[6] S. Oka, This Bulletin, **24**, 338 (1960).

content of the cell is small, the partition of the antiseptics to the lipid phase of the cell is negligibly small. However, in the case of the esters of *p*-hydroxybenzoic acid, the main part of the transferred ester is considered to exist in three states, i.e., the ester adsorbed on the solid phase of the cell, the dissolved one in the lipid phase of the cell and that in the inner cell fluid. Since the partition coefficient of the esters between oil and water is so great, it is thought that the quantity dissolved in the lipid phase can no longer be negligible.

In the present report, the role of each transferred ester under the three different states was examined in relation to the toxic effect, and it has been shown that the toxic effect mainly depends upon the adsorbed quantity of the esters, being independent of the dissolved ester in the lipid phase or in the inner cell fluid.

EXPERIMENTAL

Experiments were carried out with washed bakers' yeast, the water content of which was 65 percent of netcell weight.

The quantity of the ester transferred to the yeast cell and its toxic effect on the yeast were determined by the methods described in previous reports[6,7].

Evaluation of lipid content of yeast* The lipid content of the yeast was evaluated to be 2.6 percent after analysis of the content of glyceride determined by Fukuba's method[8] (2.28%), and that of the unsaponifiable matter determined by the usual method (0.34%).

Antiseptics As antiseptics methyl, ethyl, *n*-propyl, *n*-butyl and *n*-amyl esters of *p*-hydroxybenzoic acid were employed.

The concentration of the esters was spectro-photometrically determined in the aqueous solution. The optical extinction was measured at the wave-length of 257.5 mμ, and the molar extinction coefficient was 14,750 in any derivative of this homologue.

Determination of partition coefficient of ester between oil and water For the determination of the partition coefficient refined soy bean oil (the acid value of which was 0.21) was used instead of the yeast lipid.

A definite amount of ethanol solution of an ester, e(m-mol) as net-ester, and a definite amount of the oil, M(kg), were added to a definite amount of water containing hydrochloric acid in 0.1 percent, V(kg). (In this case the added ethanol was diluted to less than 2 percent by water.) The mixture was vigorously shaken for two minutes at 30°C, and it was then centrifuged and filtered with a dry filter paper. Then, the concentration of the ester in the filtrate, C_m(m-mol/kg-water), was determined by the optical extinction. Simultaneously, a blank test was carried out.

The partition coefficient of the ester, P, between the oil and water was evaluated from equation(1),

$$P=(e-VC_m)/MC_m \qquad (1)$$

RESULTS

Toxic effect of esters of *p*-hydroxybenzoic acid

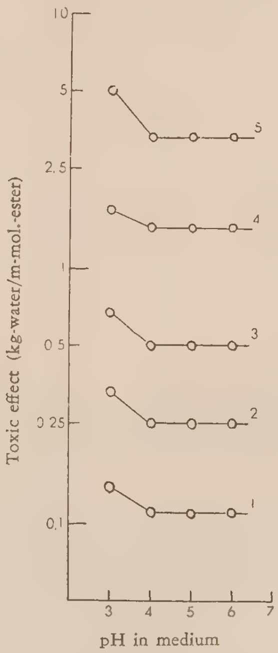

FIG. 1. Influence of pH value and Sort of Alkyl Radical on Toxic Effect of Ester of *p*-Hydroxybenzoic Acid

Koji extract containing an ester was inoculated with washed bakers' yeast in 0.0003% and incubated for 48 hours at various pH. The toxic effect of the ester was expressed by the reciprocal of the minimum concentration for inhibition of the yeast growth at 30°C. 1: methyl ester, 2: ethyl ester, 3: *n*-propyl ester, 4: *n*-butyl ester, 5: *n*-amyl ester.

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* In the case that the usual ether extraction method is used, the lipid content of the yeast is very small (0.21%). This fact indicates that the yeast lipid is mainly disparsed in the solid phase of the cell.

7) S. Oka, This Bulletin, **24**, 59 (1960).

8) H. Fukuba, N. Yamazawa and C. Inagaki, *J. Agr. Chem. Soc. Japan*, **28**, 59 (1954).

The minimum concentration of the esters required to inhibit the growth of bakers' yeast was determined in the medium of koji extract at various values of pH, and the toxic effect was expressed by the reciprocal of the inhibiting concentration as given in Fig. 1.

From this result, the toxic effect of the ester seems to be almost independent of the pH value of the culture medium, though it is somewhat higher at pH 3 than in other cases. On the other hand, the toxic effect increases at the rate of about $(2.3)^N$ with the increase of carbon number, N, in the alkyl radical of the ester.

Change of transfer ratio with suspending time From the result shown in Table I, transference of the ester seems to be practically completed within a period of two minutes.

TABLE I. CHANGE OF TRANSFER RATIO WITH SUSPENDING TIME

Suspending time (min.)	Transfer ratio (kg-water/kg-cell) Methyl ester	Propyl ester	Butyl ester
2	2.32	11.7	33.5
5	2.45	12.1	33.5
10	2.45	12.5	33.1
15	2.38	12.4	33.8
60	2.45	12.7	33.6
120	2.38	12.3	33.1
360	2.42	12.1	33.6
Initial conc. of ester (m-mol./kg-water)	3.49	0.692	0.408
Cell propn. (kg/kg-water)	0.2	0.05	0.02

Yeast cells were suspended in the buffer solution (pH 5.6) containing a definite amount of an ester at 30°C. After a definite interval of time, a part of the suspension was taken out, and the transfer ratio was determined.

Influence of pH on transfer ratio The transfer ratio of the ester seems to be independent of the pH of the medium as seen in Fig. 2, though in the case of acid antiseptics the ratio was a function of the pH value[6,7].

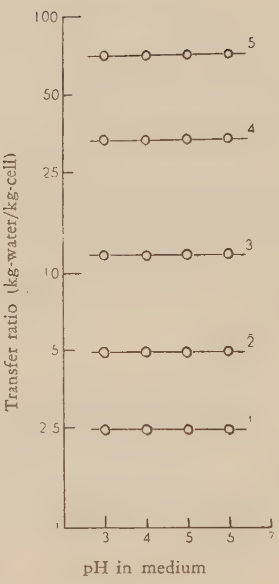

FIG. 2. Influence of pH Value and Sort of Alkyl Radical on Transfer Ratio of Ester of *p*-Hydroxybenzoic Acid

1: methyl ester, 2: ethyl ester, 3: *n*-propyl ester, 4: *n*-butyl ester, 5: *n*-amyl ester. Initial concentrations of the esters were 9.30, 3.49, 1.50, 0.692, 0.408 and 0.200 millimole per kg of water, and quantities of the yeast cell were 20, 10, 5, 2 and 1 percent, respectively. The experiment was carried out in the buffer solution at 30°C.

TABLE II. RECOVERING OF TRANSFERRED ESTER FROM CELL

	Quantity of ester in medium (μ-mol.) Methyl ester	Propyl ester	Butyl ester
Added ester	52.5	10.7	4.47
Residual in acid medium	35.2	6.68	2.60
Total in alkaline medium	51.8	11.2	4.33
Recovery (%)	98.7	104.7	96.9
Quantity of cells (g)	2.0	0.50	0.20

Yeast cells were suspended in 10 ml of the buffer solution (pH 5.6) containing a definite amount of an ester at 30°C. The transfer of the ester to the yeast cell brings about the decrease of the concentration of the ester in the medium solution. When the suspension was alkalified by addition of 10 ml of 0.1 N NaOH and allowed to stand for one hour, the added ester was completely recovered in the medium.

Recovery of transferred ester from cell The yeast was suspended in the acid medium (pH 5.6) containing an ester and allowed to stand for one hour. The suspension was subsequently alkalified with sodium hydroxide to pH 10 or higher and allowed to stand for one hour, and the total quantity of the ester in the medium was determined.

As seen in Table II, the added quantity of the ester is completely recovered in the medium. Thus, it was confirmed that no ester is de-

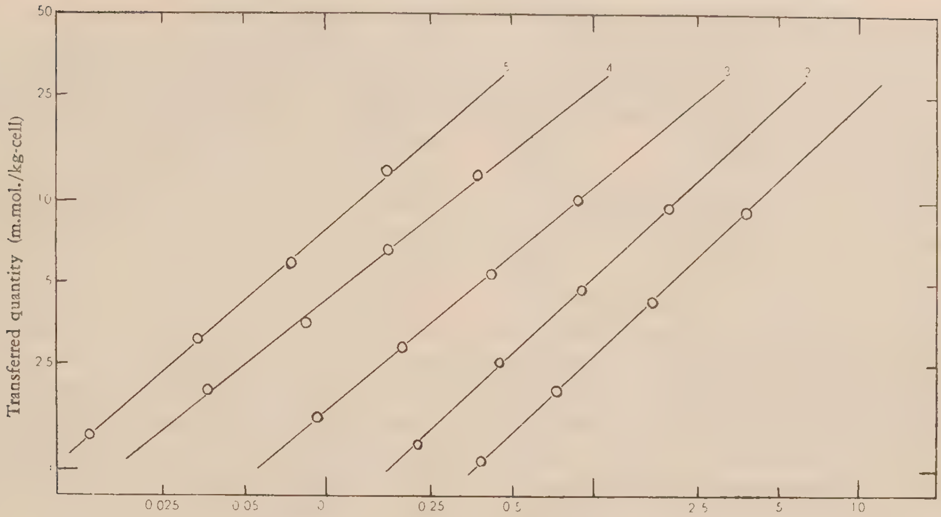

FIG. 3. Relation between Transferred Quantity and Equilibrium Concentration of Esters of *p*-Hydroxybenzoic Acid (pH 5.6)

1 : methyl ester, 2 : ethyl ester, 3 : *n*-propyl ester, 4 : *n*-butyl ester, 5 : *n*-amyl ester.

TABLE III. INFLUENCE OF OSMOTIC PRESSURE ON TRANSFER

	k (n)		Difference of k value	Dissolved quantity in cell fluid $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$
	Original buffer	Glycerin added		
Methyl ester	2.7 (0.95)	2.3 (0.87)	0.4	0.7
Ethyl ester	5.2 (0.93)	4.8 (0.88)	0.4	0.7
Propyl ester	11.2 (0.85)	11.0 (0.81)	0.2	0.3
Butyl ester	27 (0.80)	26 (0.87)	1	1.7
Amyl ester	58 (0.87)	57 (0.88)	1	1.7

The decrement of k value owing to the addition of 2 moles of glycerin per kg of water was divided by the corresponding relative decrease of water content of the yeast cell owing to the increase of the osmotic pressure in the medium (0.60). This evaluated value can be considered as the dissolved quantity of the ester in the inner cell fluid.

composed by the yeast.

Transferred quantity and equilibrium concentration The relation between the transferred quantity in the cell and the equilibrium concentration of the ester in the medium was examined at 30°C.

The plot of the logarithm of the transferred quantity versus the logarithm of the equilibrium concentration is represented by a straight line as shown in Fig. 3. This relation is expressed by equation (2)

$$a=k(C_m)^n \qquad (2)$$

where k and n are the constants of each ester, a the transferred quantity (m-mol/kg-cell), and C_m the equilibrium concentration (m-mol/kg-water). Values of k and n are given in Table III.

Influence of osmotic pressure on transfer Since the quantity of the ester dissolved in the inner cell fluid is a function of the water content of the cell and the water content is decreased by the physical action of the osmotic pressure, the transfer ratio of the ester in the concentrated solution of glycerin will decrease as in the case of the acid antiseptics[6]. However, as seen in Table III the decrement of the transfer ratio of the ester which is due to the addition of 2 mol of glycerin per kg of water is smaller than in the case of the acid antiseptics.

From the influence of the osmotic pressure on the value of k, the quantity dissolved under a condition such as that the equilibrium concentration is 1 millimole per kg water can be evaluated as described in the previous report[6].

From the evaluated dissolved quantity given in the last column of Table III, it is considered that the ratio of the dissolved quantity to the equilibrium concentration is small, being almost independent of the kind of the alkyl radical.

Partition coefficient between oil and water The partition coefficient of the ester between soy bean oil and water increases as the alkyl radical of the ester becomes higher, as shown in Table IV.

TABLE IV. PARTITION COEFFICIENT OF ESTERS BETWEEN SOY BEAN OIL AND WATER

	Partition Coefficient (30°C)
Methyl ester	7.57
Ethyl ester	24.8
Propyl ester	79.5
Butyl ester	281
Amyl ester	903

Supposing that the coefficient is essentially the same as that between the lipid of the yeast cell and water, it seems that the quantity of the ester which is dissolved in the lipid phase of the cell can not be neglected, though the lipid content of the cell is small (2.6%). In this case, the partition coefficient increases at the rate of about $(3.4)^N$ with the increase of carbon number, N, in the alkyl radical of the ester.

DISCUSSION

In the previous report[6], it has been shown that the pH value of the medium affects the adsorption of acid antiseptics on the yeast cell, and accordingly results in the variation of the toxic effect.

However, the transfer of the esters of p-hydroxybenzoic acid is independent of the pH value of the medium (Fig. 2), and the toxic effect is also independent of the pH value (Fig. 1). Accordingly, it is considered that in the case of the ester the pH value is no longer the limiting factor for the transference and the toxic effect, because the ester is not ionized in the range of the pH examined. On the other hand, the transfer ratio regularly changes with the length of the carbon chain of the alkyl radical of the ester (Fig. 2) and the toxic effect also changes in the same manner (Fig. 1). It thus appears that the kind of the alkyl radical affects the transfer of the ester, and accordingly results in the variation of the toxic effect. The transferred quantity of the ester at the minimum inhibiting concentration can be evaluated by substituting the minimum inhibiting concentration of the ester (Fig. 1) in C_m of equation (2) as given in Table V. (In this case corresponding values of k and n are given in Table III).

In connection with the transferred quantity, it has been shown that the acid antiseptic transferred to the cell exists in two states[6,7], i.e., as the adsorbed one on the solid phase of the cell and the dissolved one in the inner cell fluid. In the case of ester, the ratio of the dissolved quantity to the total transferred quantity is smaller than in the case of the acid antiseptics (Table III). Supposing that the ester can permeate into the cell in the same manner as in the case of the undissociated molecules of the acid antiseptic[7], the concentration of the ester in the inner cell fluid is expected to be equal to that of the medium (This is because the ester is not ionized in the inner cell fluid). Thus, the dissolved quantity in the inner cell fluid can be evaluated to be $0.65\,C_m$, (0.65 is the water content of the cell and C_m the equilibrium concentration in the medium). This evaluation can be justified from the dissolved quantity evaluated under the influence of the osmotic pressure (Table III).

TABLE V. ADSORBED AND DISSOLVED QUANTITIES AT MINIMUM INHIBITING CONCENTRATION OF ESTERS OF *p*-HYDROXYBENZOIC ACID

Alkyl radical of ester	Minimum conc. for inhibition of yeast growth $\left(\frac{\text{m-mol.}}{\text{kg-water}}\right)$	Transferred quantity $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$	Dissolved quantity in cell fluid $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$	Dissolved quantity in lipid of cell $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$	Adsorbed quantity $\left(\frac{\text{m-mol.}}{\text{kg-cell}}\right)$
Methyl	9	21.8	5.9	1.8	14.1
Ethyl	4	18.9	2.6	2.6	13.7
Propyl	2	20.2	1.3	4.1	14.8
Butyl	0.7	20.3	0.5	5.1	14.7
Amyl	0.3	20.1	0.2	7.0	12.9

Accordingly, the dissolved quantity at the minimum inhibiting concentration can be evaluated by multiplying the inhibiting concentration by 0.65 as given in Table V. Thus, the toxic effect is considered to be independent of the dissolved quantity in the inner cell fluid, because the dissolved quantity evaluated under a definite inhibiting condition decreases as the alkyl radical becomes higher.

Since the partition coefficient between oil and water is so great (Table IV) in the case of the ester, the dissolved quantity in the lipid phase of the cell must be taken into account, despite that the partition of the antiseptic to the lipid phase has been neglected in the author's previous studies[6,7]. By assuming that the partition coefficient given in Table IV is equal to that concerning the yeast lipid (because the yeast lipid mainly consists of glyceride), the dissolved quantity of ester in the lipid phase at the minimum inhibiting concentration can be evaluated by multiplying the lipid content of 0.026 (kg/kg-cell) by the corresponding partition coefficient and the minimum inhibiting concentration as indicated in Table V.

In the lipid phase of the cell, the dissolved quantity evaluated under the definite inhibiting condition increases as the alkyl radical becomes higher, as seen in Table V. This is because the partition coefficient increases at the rate of $(3.4)^N$, although the toxic effect increases at the rate of $(2.3)^N$. From this fact the toxic effect of the ester is also considered to be independent of the ester dissolved in the lipid phase of the cell.

By subtracting the quantity dissolved in the lipid phase and that in the inner cell fluid from the total quantity transferred, the quantity adsorbed on the solid phase of the cell can be evaluated as given in the last column of Table V. It is noteworthy that at the minimum inhibiting concentration of these esters, all of the adsorbed quantities thus evaluated are practically constant (13~15 m-mol/kg-cell), being independent of the kind of the alkyl radical.

Therefore, it can be concluded that the kind of the alkyl radical of the ester affects its adsorption on the solid phase of the cell, and thus results in the variation of the toxic effect, in other words, the adsorbed quantity is the limiting factor of the toxic effect.

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Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

III. Mechanism of Substrate Inhibition*

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Received November 13, 1959

The reaction catalyzed by crystalline yeast phosphoglyceric acid mutase is inhibited by the substrate (D-2-phosphoglyceric acid). In order to elucidate the mechanism of this substrate inhibition, detailed investigations have been performed. It is proved that the substrate inhibition in this enzyme reaction is caused by the facts that the coenzyme-binding site on the enzyme is covered by the substrate and the combination of the coenzyme with the enzyme is interfered by the substrate. Consequently, it is concluded that the substrate is a competitive inhibitor of the coenzyme.

INTRODUCTION

Phosphoglyceric acid mutase has an important role in glycolysis. Recently, studies on PGA mutase** from various sources have been advanced[1~4].

We reported some basic properties of this enzyme crystallized from baker's yeast according to a modification of the procedure of Rodwell et al.[5]. In these experiments the enzyme activities were directly measured by the polarimetric method without the aid of coupled reactions with other enzymes. In order to find out a clue to solve the mechanism of the PGA mutase reaction, it was necessary to clarify the modes of inhibitory actions by various compounds for this enzyme. In the crystalline yeast PGA mutase reaction, however, the substrate inhibition occurred[1,3], so that we could not always help obtaining complicated results in its inhibition experiments. Therefore, prior to studying the effects of inhibitors on the enzyme reaction, the mechanism of the substrate inhibition in this enzyme reaction should be researched.

In the case of PGA mutase reaction, since the structure of the substrate is similar to that of the coenzyme, it may be considered that the substrate interferes the combinations of the coenzyme with the enzyme, and *vice versa*. In this respect, we have studied the mechanism of the substrate inhibition in the enzyme reaction in detail. We have found that the substrate and the enzyme form an inactive complex when the substrate combines with the site on the enzyme to which the coenzyme is to be bound, and have proved that the substrate is a competitive inhibitor of the coenzyme.

MATERIALS AND METHODS

Three times recrystallized yeast PGA mutase, DL-2PGA (substrate) and 2,3-PGA (coenzyme) were prepared by the methods described in the previous paper[3].

The conditions of the standard assay for the enzyme activity were described before[3]. That is, the assay systems contained the following: 2.5 ml of 6.6×10^{-2} M DL-2 PGA, adjusted to pH 5.9; 0.5 ml of 10^{-3} M 2,3-PGA, adjusted to pH 5.9; 0.5 ml of 1 M sodium acetate buffer (pH 5.9); 0.5 ml of the enzyme solution containing 1 to 16 μg of the crystalline protein; and water to a final volume of 5.0 ml. The reaction was started by the addition of the enzyme solution and the mixture was incubated at 25°C for ten minutes. The reaction was terminated by the addition of 2.5 ml of 20 per cent trichloroacetic acid and then the amounts of D-3 PGA formed were determined polarimetrically, with a photoelectric polarimeter attached to a Beckman DU spectrophotometer.

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** The following abbreviations are used in this paper: PGA mutase, phosphoglyceric acid mutase; D-3PGA, D-3-phosphoglyceric acid; 2,3-PGA, D-2,3-diphosphoglyceric acid; coenzyme, 2,3-PGA; D-2PGA, D-2-phosphoglyceric acid; L-2PGA, L-2-phosphoglyceric acid; DL-2PGA, DL-2-phosphoglyceric acid.

1) V.W. Rodwell, J.C. Towne and S. Grisolia, *J. Biol. Chem.*, **228**, 875 (1957).

2) H. Edelhoch, V.W. Rodwell and S. Grisolia, *J. Biol. Chem.*, **228**, 891 (1957).

3) H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959).

4) H. Chiba and E. Sugimoto, This Bulletin, **23**, 213 (1959).

5) V.W. Rodwell, J.C. Towne and S. Grisolia, *Biochim. et Biophys. Acta*, **20**, 394 (1956).

Protein concentrations were determined by measuring the absorption at 280 mμ in a Beckman DU spectrophotometer, with an extinction coefficient $E^{1\%}_{1\,\mathrm{cm}}=13.8$ for crystalline yeast PGA mutase.

RESULTS AND DISCUSSION

The substrate inhibition occurred in the case of the yeast PGA mutase reaction.

The rate of the enzyme reaction declined with increasing the substrate concentrations (Table I).

TABLE I. EFFECT OF SUBSTRATE CONCENTRATION ON YEAST PGA MUTASE REACTION

Concn. of DL-2PGA	D-3PGA formed
mM	μ moles per 10 min.
66.0	5.25
33.0	10.00
16.5	15.10
8.3	16.00
6.6	17.30
4.1	20.50

The conditions of reaction were the same as those for the standard assay except that the concentration of the substrate was varied. 5.5 micrograms of the crystalline enzyme were used.

It is obvious that the substrate inhibition is not caused by L-2PGA coexisting in the reaction mixture[1]. Therefore, the possibility is considered that only a specific ionized form of D-2PGA may be the *active substrate*, whereas the other may be the inhibitor. As the pKa$_2'$ and pKa$_3'$ values of 2PGA are known to be 3.6 and 7.0[6], respectively, we can calculate the ratio of two ionized forms of 2PGA ([PGA$^{\equiv}$]/[PGAH$^{=}$]) at any pH.

Near the optimum pH (5.9) of this enzyme, the concentration of D-2PGAH$^{=}$ is almost inalterable, but that of D-2PGA$^{\equiv}$ is considerably variable.

TABLE II. SUBSTRATE INHIBITION AT DIFFERENT pH

Concn. of DL-2PGA	pH	Concn. of DL-2PGAH$^{=}$	Concn. of DL-2PGA$^{\equiv}$	D-3PGA formed
mM		mM	mM	μ moles per 10 min.
42	6.5	32.0	10.0	4.8
	6.0	37.8	4.2	7.4
	5.4	40.7	1.3	4.2
30	6.5	22.5	7.2	7.4
	6.0	27.0	3.0	10.0
	5.4	29.1	0.9	6.4
18	6.5	13.7	4.3	10.5
	6.0	16.2	1.8	13.4
	5.4	17.5	0.5	10.0

The conditions of reaction were the same as those for the standard assay except that the concentration of the substrate and pH were varied. 5.3 micrograms of the crystalline enzyme were used.

When we compare the degrees of the substrate inhibition at different pH, it may be possible to decide which ionized form of the substrate is the inhibitor (Table II).

If D-2PGA$^{\equiv}$ is the inhibitor, activities in the cases of nearly equal concentrations of D-2PGA$^{\equiv}$ should be similar, even if the differences among the enzyme activities caused by pH changes are considered. Besides, when one concentration of D-2PGA$^{\equiv}$ differs from the other, each enzyme activity should not be similar. Namely, the differences between the enzyme activities in the cases of the different D-2PGA$^{\equiv}$ concentrations (e.g. 0.5 and 4.3, 0.9 and 7.2 or 1.3 and 10.0×10^{-3} M of D-2PGA$^{\equiv}$) should be observed.

However, we could not obtain the results as clearly shown in Table II, to prove the above assumption that D-2PGA$^{\equiv}$ is the inhibitor. Consequently, D-2PGA$^{\equiv}$ is not the inhibitor.

Therefore, we can assume the following three cases for the substrate inhibition in this enzyme reaction.

Active Substrate	D-2 PGA$^{\equiv}$	D-2 PGA$^{\equiv}$	D-2 PGAH$^{=}$
Inhibitor	D-2 PGAH$^{=}$	i	i

Here, the *active substrate* is D-2PGA$^{\equiv}$ or D-2PGAH$^{=}$ and the inhibitor is D-2PGAH$^{=}$ or an unknown inhibitor expressed in i. In these cases, it is assumed that the *active substrate* and the coenzyme are bound to the different sites on the enzyme; and one has no tendency to be attached to the site reserved for the other.

We can regard the substrate inhibition as the special case where the inhibitor is always added to the substrate.

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[6] F. Wold and C. E. Ballou, *J. Biol. Chem.*, **227**, 301 (1957).

We can also regard the general case where the substrate inhibition does not occur as the case where no inhibitor is added to the substrate.

In the general case, the following equation (1) is obtained at the particular pH value and substrate concentration[7].

$$v=\frac{k_3[\mathrm{E}]_0\mathrm{A}\ [\mathrm{S}]_0\ [\mathrm{C}]_0}{(\mathrm{Ks}+\mathrm{A}[\mathrm{S}]_0)\ (\mathrm{Kc}+[\mathrm{C}]_0)}\ \cdots\cdots\ (1)$$

where $[\mathrm{E}]_0$, $[\mathrm{S}]_0$ and $[\mathrm{C}]_0$ are the total concentrations of the enzyme, the substrate and the coenzyme added, respectively, Ks and Kc are the dissociation constants of the enzyme-*active substrate* complex and the enzyme-coenzyme complex, respectively, and k_3 is the rate constant. $\mathrm{A}[\mathrm{S}]_0$ is the concentration of the *active substrate* at the particular pH. Since the concentration of the substrate added and pH are constant, when the constant term is replaced with Z, the equation (1) is rearranged to

$$v=\frac{\mathrm{Z}[\mathrm{C}]_0}{\mathrm{Kc}+[\mathrm{C}]_0}\ \cdots\cdots\cdots\cdots\cdots(2)$$

$$\mathrm{Z}=\frac{k_3[\mathrm{E}]_0\ \mathrm{A}[\mathrm{S}]_0}{\mathrm{Ks}+\mathrm{A}[\mathrm{S}]_0}$$

In the case of the substrate inhibition where we assume that the inhibitor is always added to the substrate, the competitive (a) and noncompetitive inhibition (b) or (b′) for the coenzyme give the equation (3) and (4) or (4′), respectively. "Competitive" means that the inhibitor is bound to the site to which the coenzyme is to be bound and interferes the combination of the coenzyme with the enzyme. "Noncompetitive" means no binding of the inhibitor to the site to which the coenzyme should be bound. In the case of (b), the inhibitor is not bound to the both sites. Namely, the inhibitor is a noncompetitive inhibitor of the both compounds. In the case of (b′), the inhibitor can not be bound to the coenzyme-binding site, but can be bound to the *active substrate*-binding site. Namely, the inhibitor is a noncompetitive inhibitor of the coenzyme, and a competitive inhibitor of the *active substrate.*

$$\text{(a)}\quad \frac{1}{v}=\frac{1}{\mathrm{Z}}+\frac{\mathrm{Kc}}{\mathrm{Z}[\mathrm{C}]_0}\left(1+\frac{\mathrm{B}[\mathrm{S}]_0}{\mathrm{Ki}}\right)\ ..(3)$$

$$\text{(b)}\quad \frac{1}{v}=\frac{1}{\mathrm{Z}}\left(1+\frac{\mathrm{Kc}}{[\mathrm{C}]_0}\right)\left(1+\frac{\mathrm{B}[\mathrm{S}]_0}{\mathrm{Ki}}\right)\ ..(4)$$

$$\text{(b}'\text{)}\quad \frac{1}{v}=\frac{1}{\mathrm{Z}}\left(1+\frac{\mathrm{Kc}}{[\mathrm{C}]_0}\right)\times\left(1+\frac{\mathrm{Ks}\ \mathrm{B}[\mathrm{S}]_0}{(\mathrm{Ks}+\mathrm{A}[\mathrm{S}]_0)\ \mathrm{Ki}}\right)\ \ldots.(4')$$

Here, the inhibitor concentration can be expressed in $\mathrm{B}[\mathrm{S}]_0$ under the specific conditions where $[\mathrm{S}]_0$ and pH are constant, and Ki is the corresponding dissociation constant. In the cases of (a) and (b) or (b′), when the reciprocal of velocity is plotted against the reciprocal of the coenzyme concentration, a straight line is obtained.

But in the case of (a), the intercept on $1/[\mathrm{C}]_0$ axis is $-\mathrm{Ki}/\mathrm{Kc}(\mathrm{Ki}+\mathrm{B}[\mathrm{S}]_0)$ whose value is dependent on the substrate concentrations. If the reaction rates are tested over as wide a range of the coenzyme concentrations as possible at two different substrate concentrations, $[\mathrm{S}_1]_0$ and $[\mathrm{S}_2]_0$, the resultant straight lines cut one another. At the intersection, $1/[\mathrm{C}]_0$ is equal to $-\mathrm{KiKs}/\mathrm{Kc}(\mathrm{KiKs}-\mathrm{AB}[\mathrm{S}_1]_0[\mathrm{S}_2]_0)$ which is dependent on the substrate concentrations.

In the cases of (b) or (b′), the intercept on $1/[\mathrm{C}]_0$ axis is $-1/\mathrm{Kc}$ which is constant at any substrate concentrations. That is, the straight lines corresponding to several different substrate concentrations meet only at a point on the base line. Therefore, it can be decided that the mode of the substrate inhibition is either "competitive" or "noncompetitive" for the coenzyme, when plotting the reciprocal of velocity against the reciprocal of the coenzyme concentration at the different fixed values of the substrate concentrations.

From the results shown in Fig. 1, it is obvious that the mode of the substrate inhibition in the case of the yeast PGA mutase reaction is "competitive" for the coenzyme. Namely, the substrate combines with the coenzyme-binding

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[7] K. J. Laidler and I.M. Socquet, *J. Phys. Colloid. Chem.*, **54**, 530 (1950).

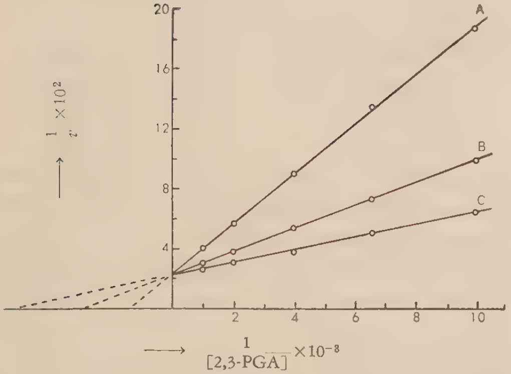


FIG. 1. Effect of Coenzyme Concentration on Yeast PGA Mutase Reaction at Various Substrate Concentrations.

The conditions of reaction were the same as those for the standard assay except that the coenzyme concentration was varied at the different concentrations of the substrate. The concentrations of DL-2PGA were 6.6×10^{-2} M (A), 3.3×10^{-2} M (B) and 1.6×10^{-2} M (C), respectively. 5.5 micrograms of the crystalline enzyme were used. v is expressed as micromoles of D-3PGA formed per ten minutes.

site on the enzyme and thus affects the combination of the coenzyme with the enzyme.

An intercept on $1/v$ axis is

$$1/Z,\ \frac{K_s+A[S]_0}{k_3[E]_0A[S]_0},$$

which is also dependent upon the substrate concentrations. In the case of $K_s \ll A[S]_0$, however, an intercept on $1/v$ axis is approximately equal to $1/k_3[E]_0$. Consequently, even when the substrate concentrations are varied under such a condition described above, all the straight lines cut $1/v$ axis at the fixed point. As obviously shown in Fig. 1, since every straight line meets in a point on $1/v$ axis, it is supposed that Ks is greatly smaller than $[S]_0$ used.

$K_c(1+B[S]_0/K_i)$ corresponds to the apparent Michaelis constant for the coenzyme.

TABLE III. APPARENT MICHAELIS CONSTANTS FOR COENZYME

Concn. of DL-2PGA (D-2PGA)	Apparent Kc $K_c\left(1+\frac{B[S]_0}{K_i}\right)$
mM	mM
66.0 (33.0)	0.647
33.0 (16.5)	0.330
16.0 (8.0)	0.167

Apparent Kc values were determined from the results shown in Fig. 1.

As shown in Table III, the apparent Kc for the coenzyme is variable at the different substrate concentrations. In the previous paper[3)], we reported the apparent Kc for the coenzyme to be 3.3×10^{-4} M, which was obtained under the conditions of the standard assay. From these values in Table III, Kc can be calculated. The Kc value is 1.3×10^{-5} M or less than that.

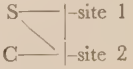
In addition to the cases described above where the *active substrate* and the coenzyne combine with the enzyme independently of each other, the other cases should be considered.

If the *active substrate* (or the coenzyme), however, can combine with the *active substrate*-binding site and also with the coenzyme-binding site, the *active substrate* (or the coenzyme) becomes a competitive inhibitor of the coenzyme (or the *active substrate*). Therefore, in addition to the cases (a) and (b) or (b′), the following cases, (c) and (d), should be considered to study the mechanism of the substrate inhibition. Since D-2PGA$^{\equiv}$ is not the inhibitor, as shown in Table II, there appears no compound to become the *active substrate* and the inhibitor at the same time except D-2PGAH$^{=}$.

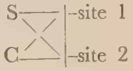
Active Substrate D-2 PGAH$^{=}$

Inhibitor D-2 PGAH$^{=}$

(c)



(d)



In the case of (c), the *active substrate* can combine with the site 1 on the enzyme, while the coenzyme can combine only with the site 2. But the *active substrate* can also combine with the site 2 reserved for the coenzyme. That is, the *active substrate* is a competitive inhibitor of the coenzyme. The enzyme reaction can be represented by the following reaction sequences.

$$E + S \rightleftharpoons ES \qquad K_s$$

$$ES + C \rightleftharpoons ESC \qquad K_c$$

$$ESC \xrightarrow[k_3]{} EC + \text{Product}$$

$$E + C \rightleftharpoons EC \qquad K_c$$

$$EC + S \rightleftharpoons ECS \qquad K_s$$

$$ECS \xrightarrow[k_3]{} EC + \text{Product}$$

$$E + S \rightleftharpoons ES' \qquad K_{si}$$

$ES + S \rightleftharpoons ESS'$	Ksi
$ES' + S \rightleftharpoons ES'S$	Ks

where E is the enzyme, S is the *active substrate* and C is the coenzyme, ES is the enzyme whose site 1 is covered by the *active substrate* (effective enzyme-substrate complex) and Ks the corresponding dissociation constant; similarly, EC (effective enzyme-coenzyme complex) and Kc are for the coenzyme on the site 2. ESC(or ECS) is the active ternary complex of the enzyme. ES′ is the enzyme whose coenzyme-binding site (site 2) is covered by the *active substrate* (ineffective complex) and Ksi the corresponding dissociation constant. ESS′ (or ES′S) is the enzyme whose sites 1 and 2 are covered by the *active substrate* (ineffective complex).

The total amounts of the enzyme, $[E]_0$, is as follwos,

$$[E]_0=[E]_{free}+[ES]+[ESC]+[EC]+[ES']+[ESS']$$

where the concentrations are those in equilibrium. The reaction velocity is expressed in

$$v=\frac{k_3[E]_0\ A\ [S]_0\ [C]_0}{(Ks+A[S]_0)\left[Kc\left(1+\frac{A[S]_0}{Ksi}\right)+[C]_0\right]} \quad ..\ (5)$$

Taking the reciprocal of both sides

$$\frac{1}{v}=\frac{Kc}{k_3[E]_0[C]_0}\left(1+\frac{Ks}{A[S]_0}\right)\left(1+\frac{A[S]_0}{Ksi}\right)$$
$$+\frac{1}{k_3[E]_0}\left(1+\frac{Ks}{A[S]_0}\right) \quad \cdots\cdots\cdots\ (6)$$

Hence, in this case, if $1/v$ is plotted against $1/[C]_0$, keeping $A[S]_0$ constant, a straight line is obtained.

$$\frac{1}{v}=\frac{1}{Z}+\frac{Kc}{Z[C]_0}\left(1+\frac{A[S]_0}{Ksi}\right) \quad \cdots\cdots\ (7)$$

$$Z=\frac{k_3\ [E]_0\ A[S]_0}{Ks+A[S]_0} \quad \text{(constant)}$$

The intercept of this line on $1/[C]_0$ axis is $-Ksi/Kc(Ksi+A[S]_0)$ whose value is dependent on the substrate concentrations. If $[S_1]_0$ and $[S_2]_0$ are arbitrarily chosen, the straight lines corresponding to two different substrate concentrations cut one another. At the intersection, $1/[C]_0$ is equal to $-KsiKs/Kc(KsiKs-A^2[S_1]_0[S_2]_0)$, whose value is dependent on $[S]_0$. The case of (c) is similar to that of (a). It is clear that the case of (c) is also suitable to the mechanism of the substrate inhibition in this enzyme reaction.

In the case of (d), the *active substrate* can combine with the site 1, and the coenzyme with the site 2, besides the *active subsrtate* can combine with the site 2 and the coenzyme with the site 1. That is, the *active substrate* and the coenzyme interfere the respective combinations with the enzyme.

We can represent the system by the following reaction.

$E + S \rightleftharpoons ES$	Ks
$ES + C \rightleftharpoons ESC$	Kc
$ESC \xrightarrow[k_3]{} EC + \text{Product}$	
$E + C \rightleftharpoons EC$	Kc
$EC + S \rightleftharpoons ECS$	Ks
$ECS \xrightarrow[k_3]{} EC + \text{Product}$	
$E + S \rightleftharpoons ES'$	Ksi
$E + C \rightleftharpoons EC'$	Kci
$ES' + C \rightleftharpoons ES'C'$	Kci
$EC' + S \rightleftharpoons EC'S'$	Ksi
$ES' + S \rightleftharpoons ES'S$	Ks
$ES + S \rightleftharpoons ESS'$	Ksi
$EC' + C \rightleftharpoons EC'C$	Kc
$EC + C \rightleftharpoons ECC'$	Kci

where EC′ is the enzyme whose site 1 is covered by the coenzyme (ineffective enzyme-coenzyme complex) and Kci the corresponding dissociation constant. ECC′ (or EC′C) is the enzyme whose sites 1 and 2 are covered by the coenzyme (ineffective complex). ES′C′ (or EC′S′) is the enzyme whose site 1 is covered by the coenzyme and site 2 by the *active substrate* (inactive ternary complex).

In this system,

$$[E]_0=[E]_{free}+[ES]+[EC]+[ESC]+[ES']+[EC']$$
$$+[ESS']+[ECC']+[ES'C']$$

$$v=\frac{k_3[E]_0A[S]_0[C]_0}{KcKs\left(1+\frac{A[S]_0}{Ks}+\frac{[C]_0}{Kci}\right)\left(1+\frac{[C]_0}{Kc}+\frac{A[S]_0}{Ksi}\right)} \quad \cdots\cdots\cdots(8)$$

Keeping $A[S]_0$ constant, the equation (8) is rearranged to

$$\begin{aligned}\frac{1}{v}=&\frac{\mathrm{Kc}}{\mathrm{k_3[E]_0[C]_0}}\left(1+\frac{\mathrm{Ks}}{\mathrm{A[S]_0}}+\frac{\mathrm{Ks}}{\mathrm{Ksi}}+\frac{\mathrm{A[S]_0}}{\mathrm{Ksi}}\right)\\&+\frac{1}{\mathrm{k_3[E]_0}}\left(1+\frac{\mathrm{Ks}}{\mathrm{A[S]_0}}+\frac{\mathrm{KcKs}}{\mathrm{KciKsi}}+\frac{\mathrm{KcKs}}{\mathrm{KciA[S]_0}}\right)\\&+\frac{\mathrm{Ks[C]_0}}{\mathrm{k_3[E]_0KciA[S]_0}}\cdots\cdots\cdots\cdots\cdots(9)\end{aligned}$$

In this case, it is clear that we can not obtain a straight line by plotting $1/v$ against $1/[\mathrm{C}]_0$, so that the case (d) should be excluded.

Hereupon, the mechanism of the substrate inhibition in the yeast PGA mutase reaction may be subjected to the mode represented in the case of (a) or (c).

In the substrate inhibition in this enzyme reaction, the uncompetitive inhibition[8) need not be considered, because, as the apparent Kc (slope/intercept) decreases with the increase of the substrate concentration in the uncompetitive case, our experimental results in which the apparent Kc increases with the increase of the substrate concentration can not be explained by the mechanism of the uncompetitive inhibition.

When we can identify one of the ionized forms of D-2PGA with the *active substrate* or the inhibitor, it will be clearly solved which of the cases ((a) and (c)) is suited to the mechanism of the substrate inhibition in the yeast PGA mutase reaction.

The mode of the substrate inhibition, however, has been elucidated, that is, the combination of the coenzyme with the enzyme is interfered by that of the substrate with the coenzyme-binding site.

Consequently, in the case of the yeast PGA mutase reaction, it has been ascertained that the substrate is a competitive inhibitor of the coenzyme.

SUMMARY

The mechanism of the substrate inhibition in the yeast PGA mutase reaction has been studied in detail.

It is concluded that the case of (a) or (c) is suitable to the mode of this substrate inhibition.

It is confirmed that the coenzyme competes with the substrate for the coenzyme-binding site on the enzyme; the substrate is a competitive inhibitor of the coenzyme.

The Michaelis constant for the coenzyme (Kc) is calculated to be 1.3×10^{-5} M or less than that.

Acknowledgement. The authors wish to express their thanks to Prof. H. Mitsuda of Kyoto University for his kind advice and suggestion throughout the course of this work.

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8) E.R. Ebersole, C. Guttentag and P. W. Wilson, *Arch. Biochem.*, **3**, 399 (1944).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 424～427, 1960]

Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

IV. Mechanism of Some Inhibitions*

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Received November 13, 1959

Various compounds reveal the inhibitory actions for yeast phosphoglyceric acid mutase. In order to clarify the mechanism of this enzyme reaction, it is necessary to understand the behaviors of the inhibitors in relation to the unavoidable substrate inhibition. According to the result, it is confirmed that Zn^{++} and pyrophosphate are competitive inhibitors of the coenzyme. Moreover, the mechanism of the fluoride inhibition has been investigated under the several conditions in detail.

INTRODUCTION

PGA mutase** reaction is inhibited by various compounds. Some studies on effects of the inhibitors on the muscle and yeast PGA mutase reactions were reported by Rodwell et al.1), and on the muscle PGA mutase reaction by Cowgill and Pizer2).

We have also described the behaviors of the inhibitors in the yeast PGA mutase reaction3). As already reported, several metallic ions, chelating agents (pyrophosphate and citrate etc.) and substrate analogues (phosphorylenolpyruvate etc.) revealed the inhibitory actions, although SH-inhibitors (*p*-chloromercuribenzoate etc.) had no effect on the enzyme. The inhibitions by some metallic ions which could inhibit the enzyme activity only in their higher concentrations might be caused by salt effect.

Since the inhibition caused by the other metallic ions inhibiting the activity even in their lower concentrations might not be due to mercaptide formation, it was assumed that their inhibitory actions occured by a specific mechanism.

As, in the case of the inhibitions by chelating agents, there was found no contribution of metals to the enzyme activity, they appeared to behave specially. Hereupon, we have investigated the inhibition mechanisms of the poisonous metallic ions at lower concentrations and chelating agents in detail, respectively. It has been clearly shown that the rate of the inhibitory reactions is influenced by the interrelation between those compounds and the coenzyme.

On the other hand, yeast PGA mutase was especially inhibited by fluoride ion3). Studies on the fluoride inhibition of muscle PGA mutase were reported by Cowgill and Pizer2). In the case of yeast PGA mutase, chelating agents which were given at higher concentrations than fluoride protected the enzyme from the inhibitory action of fluoride. In order to elucidate the mechanism of the fluoride inhibition, further studies have been performed.

MATERIALS AND METHODS

Three times recrystallized PGA mutase, DL-2PGA and 2,3-PGA were prepared by methods described in the previous paper3).

The conditions of the standard assay for the activity were described before[3]. Protein concentrations were determined as described[3].

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** The following abbreviations are used in this paper: PGA mutase, phosphoglyceric acid mutase; D-3PGA, D-3-phosphoglyceric acid; DL-2PGA, DL-2-phosphoglyceric acid; 2,3-PGA, D-2,3-diphosphoglyceric acid; EDTA, ethylenediaminetetraacetic acid; coenzyme, 2,3-PGA.

1) V.W. Rodwell, J.C. Towne and S. Grisolia, *J. Biol. Chem.*, **228**, 875 (1957).

2) R.W. Cowgill and L.I. Pizer, *J. Biol. Chem.*, **223**, 885 (1956).

3) H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959).

RESULTS AND DISCUSSION

Inhibitory mode of Zn^{++} When the studies on the behaviors of the inhibitors are performed, the effect of the substrate inhibition on the results obtained in the inhibition experiments should be always considered. As described in the preceding paper[4], the substrate inhibition occurs in the enzyme reaction according to the mode of case (a) or (c). That is, the substrate is a competitive inhibitor of the coenzyme. Therefore, the rate equation in this enzyme reaction is given as follows.

$$v=\frac{k_3[E]_0A[S]_0[C]_0}{(Ks+A[S]_0)\left[Kc\left(1+\frac{B[S]_0}{Ki}\right)+[C]_0\right]} \quad ....(1)$$

Here, the expression of each term is the same as described in the preceding paper[4]. If the inhibitor added is a competitive inhibitor of the coenzyme, equation (2) is obtained.

$$v=\frac{k_3[E]_0A[S]_0[C]_0}{(Ks+A[S]_0)\left[Kc\left(1+\frac{B[S]_0}{Ki}+\frac{[I]}{K_I}\right)+[C]_0\right]} \quad ....(2)$$

in which $[I]$ is the concentration of a competitive inhibitor of the coenzyme and K_I is the corresponding dissociation constant. Under such conditions that $[S]_0$ and pH are constant, when the constant term is replaced with Z, the equation (2) is rearranged to

$$v=\frac{Z[C]_0}{Kc\left(1+\frac{B[S]_0}{Ki}+\frac{[I]}{K_I}\right)+[C]_0} \quad ....(3)$$

$$Z=\frac{k_3[E]_0A[S]_0}{Ks+A[S]_0}$$

Taking the reciprocal of both sides,

$$\frac{1}{v}=\frac{1}{Z}+\frac{Kc}{Z[C]_0}\left(1+\frac{B[S]_0}{Ki}+\frac{[I]}{K_I}\right) \quad ....(4)$$

Equation (4) shows that in the presence of a competitive inhibitor of the coenzyme a straight line can be obtained when $1/v$ is plotted against $1/[C]_0$.

In this case this line cuts the vertical line at a point giving 1/Z. Therefore, even if experiments are carried out at two or more different concentrations of the inhibitor, the resultant lines have the same intercept on $1/v$ axis but different slopes. That is, these lines meet at a point on $1/v$ axis which gives 1/Z. The value of intercept on $1/v$ axis obtained in the absence of the inhibitor is the same as that in the presence of the inhibitor. If the inhibitor added is a noncompetitive inhibitor of the coenzyme, the straight lines obtained corresponding to the different inhibitor concentrations do not meet at a point on $1/v$ axis, but they meet in a point on $1/[C]_0$ axis. Thus, in order to elucidate the inhibition mechanism of the metallic ions affecting the enzyme activity in their lower concentrations, Zn^{++} was chosen.

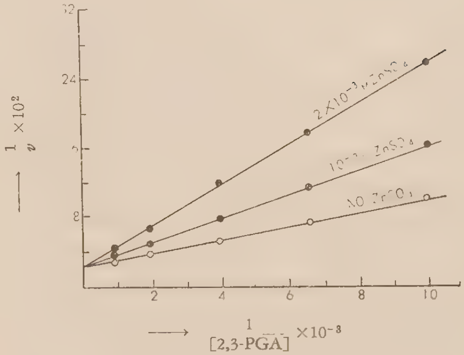

FIG. 1. Effect of Coenzyme Concentration on Inhibitory Action of Zn^{++}.

The conditions of reaction were the same as those for the standard assay except that the coenzyme concentration was varied and Zn^{++} was added. 5.5 micrograms of the crystalline enzyme were used. v is expressed as micromoles of D-3PGA formed per ten minutes.

In the presence of Zn^{++} (2×10^{-3} M and 10^{-3} M), on plotting $1/[C]_0$ versus $1/v$, we obtained the results as shown in Fig. 1.

These facts show clearly that Zn^{++} is a competitive inhibitor of the coenzyme.

Inhibitory mode of pyrophosphate. We studied the effect of pyrophosphate, which was chosen as a representative of chelating agents, on the combination of the coenzyme with the enzyme.

As shown in Fig. 2, pyrophosphate also acts as a competitive inhibitor of the coenzyme.

It is interesting, however, that Zn^{++} as cation

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[4] H. Chiba, E. Sugimoto and M. Kito, This Bulletin, **24**, 418 (1960).

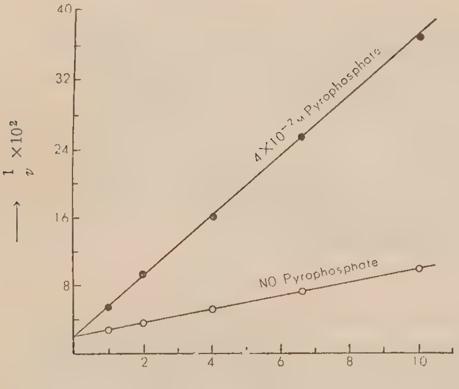

FIG. 2. Effect of Coenzyme Concentration on Inhibitory Action of Pyrophosphate.

The conditions of reaction were the same as those for the standard assay except that the coenzyme concentration was varied and pyrophosphate was added. 5.5 micrograms of the crystalline enzyme were used. *v* is expressed as micromoles of D-3PGA formed per ten minutes.

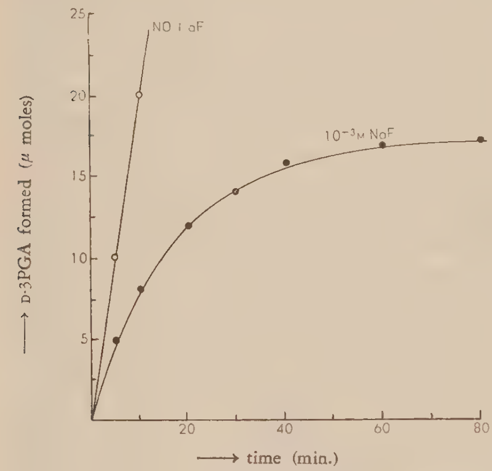

FIG. 3. Time Course of PGA Mutase Reaction in the Presence of Fluoride.

The conditions of reaction were the same as those for the standard assay except that fluoride was added. Eleven micrograms of the crystalline enzyme were used.

and pyrophosphate as anion compete with the coenzyme for the coenzyme-binding site, respectively. These facts suggest that the binding site of the coenzyme on the enzyme may be composed of three attaching points.

Inhibitory mode of fluoride. Fluoride inhibited the enzyme in a special manner. But this inhibition was prevented by the addition of EDTA (or other chelating agents) whose concentration was higher than that of fluoride[3].

TABLE I. FLUORIDE INHIBITION UNDER VARIOUS CONDITIONS

Group	Preincubation	Start	Inhibition (%)
I	S C F	E	55
	S C F EDTA	E	0
	S C F —EDTA—	E	0
	S C F	E EDTA	0
II	E C F	S	55
	E C F EDTA	S	0
	E C F —EDTA—	S	0
	E C F	S EDTA	0
III	E S F	C	100
	E S F EDTA	C	0
	E S F —EDTA—	C	100
	E S F	C EDTA	100

Preincubation was carried out for one hour at 25°C. The final concentration of each compound was as follows: S(DL-2PGA), 3.3×10^{-2} M; C(2,3-PGA), 10^{-4} M; F(NaF), 10^{-3} M; and EDTA, 10^{-2} M. Eleven micrograms of the crystalline enzyme were used. (—EDTA—) meant that EDTA was added to the mixture (e.g. in group I, S, C and F) after its preincubation had been performed for thirty minutes and then preincubation were continued for further thirty minutes. After the reaction was started, the amounts of D-3PGA formed in ten minutes were determined under the conditions of the standard assay.

The time course of the reaction is shown in Fig. 3. The line obtained in the presence of fluoride is extremely curved in comparison with that in the absence of fluoride. These results suggest that some period is required before fluoride at a given concentration reveals its maximum inhibitory action for the enzyme. In this respect, fluoride was preincubated with the enzyme reaction mixture under different conditions, and its inhibitory effects were studied. Moreover, the effects of EDTA on the fluoride inhibition were investigated. These results are shown in Table I.

In order to compare the activities under several conditions of preincubation, the amounts of D-3PGA formed in ten minutes were determined. In the group I, when the reaction was started by the addition of enzyme into the mixture of the substrate, the coenzyme and fluoride which was preincubated for one hour at 25°C, fluoride inhibited the enzyme activity 55 per cent. In the group II, when started by the addition of the substrate into the mixture of the enzyme, the coenzyme and fluoride preincubated, the same results were obtained. In these cases, however, EDTA protected the enzyme from the fluoride inhibition, whenever it might be added into the preincubated mixture. In group III, when the reaction was started by the addition of the coenzyme to the preincubated mixture of the enzyme, the substrate and fluoride, the 100 per cent inhibition was found. In this case EDTA showed the protecting effect only when it was added to the mixture before (or at the same time) the enzyme, the substrate and fluoride were preincubated. EDTA added after preincubation of the reactants described above, however, showed no effect. From these facts, it appears that the complete inhibition by fluoride is found only when the enzyme, the substrate and fluoride were contacted together over a long period.

It was reported that fluoride inhibited enolase[5] and phosphoglucomutase[6] by the formation of magnesium-fluoro-phosphate complex. But we could not find the requirements of Mg^{++} and phosphate for the fluoride inhibition of this enzyme. Although there seems to be a contribution of a metallic ion except Mg^{++} to the fluoride inhibition, the fluoride inhibition could not be ceased by treating the substrate with Dowex 50W. The protecting effects of chelating agents, however, still suggest that the fluoride inhibition is dependent on metallic ions. Hence this enzyme may form an inactive complex with fluoride and metallic ions which are in such trace amounts as not to be excluded even through the ion exchange resin. EDTA (or other chelating agents) appears to prevent the formation of this inactive complex by trapping trace amounts of metallic ions. On the other hand, as shown in the case of group III, since EDTA had no protecting effect, it is assumed that the inactive complex is formed irreversibly.

SUMMARY

In yeast PGA mutase reaction, the inhibition by Zn^{++} is competitive for the coenzyme.

Pyrophosphate also acts as a competitive inhibitor of the coenzyme.

It is found that the enzyme is protected by EDTA from the fluoride inhibition when EDTA is added into the reaction mixture before the enzyme is preincubated with the substrate and fluoride.

It seems that fluoride irreversibly forms an inactive complex with the enzyme in the presence of the substrate.

Acknowledgement The authors wish to express their thanks to Prof. H. Mitsuda of Kyoto University for his kind advice throughout the course of this work.

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[5] O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941~42).

[6] V.A. Najjar, *J. Biol. Chem.*, **175**, 281 (1948).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 4, Suppl., p. 428~433, 1960]

Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

V. Separation and Crystallization of Each Component from Crystalline Phosphoglyceric Acid Mutase*

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Received November 13, 1959

Crystalline yeast phosphoglyceric acid mutase is composed of several components electrophoretically and these components have the different specific activities. Therefore, in order to study the differences among the individual components, it is necessary to isolate each component in a large quantity. For this purpose, the special vertical zone electrophoresis apparatus with $Hg\text{-}Hg_2Cl_2$ reversible electrodes has been constructed and by using it a large amount of each component has been separated. Moreover, each component separated has been crystallized. The crystalline forms of the individual components are the same.

INTRODUCTION

Crystalline yeast PGA mutase** was found to be composed of several components by means of boundary electrophoretic analysis[1,2], and so it was necessary to decide which of them was PGA mutase itself.

For this purpose, performing the horizontal zone electrophoresis whose supporting material was made of amylose-starch-hyflo super-cel, we succeeded in the separation of each component[2]. As the result, we could decide that every component was PGA mutase protein having a different specific activity from others. It is very significant and interesting that this crystalline enzyme was still composed of several components even after crystallizations were repeated some times. In order to clarify the reason in the field of protein and enzyme chemistry why a specific activity or an electrophoretic mobility of each component differs from others, it is necessary to obtain each component in a large quantity.

As previously reported[2], we had a good result in a separation of each component but its amount was very small, because we could hardly apply a large amount of the enzyme protein in the case of the horizontal zone electrophoresis.

Hereupon, we have accomplished the construction of the special vertical zone electrophoresis apparatus by which we have succeeded in the separation of a large quantity of each component. Besides, we have succeeded in the crystallization of each component separated.

MATERIALS AND METHODS

Three times recrystallized yeast PGA mutase, DL-2PGA and 2,3-PGA were prepared by methods described in the previous paper[3].

The assay conditions of the enzyme activity were the same as described before[3]. Protein was determined spectrophotometrically by measuring the light absorption at 280 mμ as described before[3].

Boundary electrophoretic analysis was performed at 0.7°C with a Tiselius apparatus equipped with a diagonal slit device of Svensson[4]. Mobilities were calculated for the descending boundaries from conductivity values determined at 0.7°C.

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* The outline of this paper was reported in This Bulletin, **23**, 340 (1959) as a short communication.

** The following abbreviations are used in this paper: DL-2PGA, DL-2-phosphoglyceric acid; 2,3-PGA, D-2,3-phosphoglyceric acid; D-3PGA, D-3-phosphoglyceric acid; PGA mutase, phosphoglyceric acid mutase; coenzyme, 2,3-PGA.

1) H. Edelhoch, V.W. Rodwell and S. Grisolia, *J. Biol. Chem.*, **228**, 891 (1957).

2) H. Chiba and E. Sugimoto, This Bulletin, **23**, 213 (1959).

3) H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959).

4) H. Svensson, *Kolloid. Z.*, **87**, 181 (1939).

Vertical zone electrophoresis was carried out in a cold room at 6°C by the following procedures.

Apparatus and Technique of Vertical Zone Electrophoresis

We constructed the vertical zone electrophoresis apparatus by improving that of Porath[5]. Its electrode is the $Hg\text{-}Hg_2Cl_2$ reversible electrode, which makes it possible to perform run of long duration. The whole apparatus system is semi-open in order to compensate unavoidable electroosmotic flow automatically which occurs in an electrophoresis column during run.

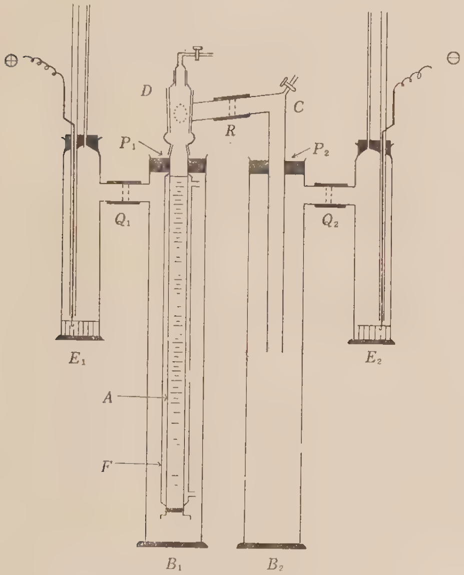

FIG. 1. Construction of Vertical Zone Electrophoresis Apparatus

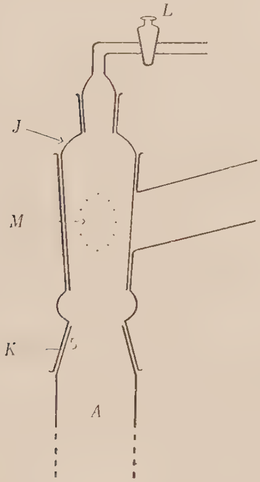

FIG. 2. Top-piece (D)

Construction of the apparatus.

As shown in Fig. 1, the apparatus consists of an electrophoresis column tube (*A*), two buffer cylinders (B_1, B_2), a connecting tube (*C*), a top-piece (*D*) and two electrode vessels (E_1, E_2). *A* is a glass tube (60×3 cm) with a water jacket (*F*) to cool a column during elution. At the lower end of *A* a glass filter is attached to support the cellulose bed. *A* is to be immersed into a cylindrical outer tube (B_1) filled with the conducting buffer solution.

As shown in Fig. 2, the upper end of *A*, being terminated by a ground joint (*K*), is jointed to a top-piece (*D*) with a side arm. A ground joint (*J*) in *D* has an opening (*M*) which is turned to coincide with an opening of the side arm. The capacity of B_1 (or B_2)

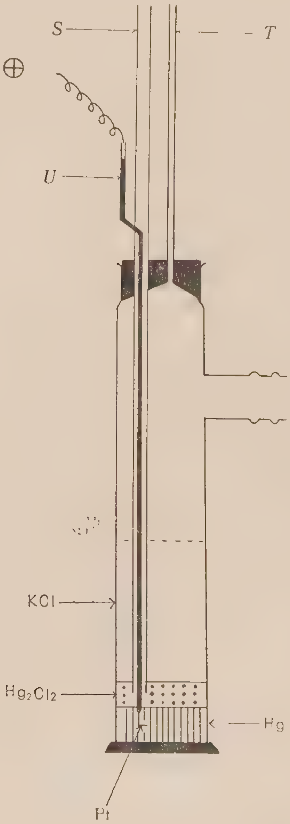

FIG. 3. Electrode Vessel

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5) J. Porath, *Acta Chem. Scand.*, **8**, 1813 (1954).

is about 6 l and a side arm near the top of B_1 (or B_2) is connected to E_1 (or E_2). The connection between B_1 and B_2 is performed via A, D and C. A and C are kept in position by rubber stoppers (P_1, P_2) on B_1 and B_2. The joints between D and C, E_1 and B_1, and E_2 and B_2 are performed by rubber tubings (R, Q_1, Q_2). The electrode vessel (E_1 or E_2) whose volume is about one liter has a construction shown in Fig. 3.

An electrode is a Hg-Hg_2Cl_2 reversible one. The electrode lead (U) is made of a glass tube in which mercury is filled. At the lower end of U is sealed platinum wire which is to be immersed into mercury zone. From the upper end of a glass tube (S), saturated potassium chloride solution is poured into E_1 (or E_2). S also has a role to support U. The diameters of the tubes T and S are very small as compared with that of the electrophoresis column tube. Therefore, electroosmotic flow occurring during run is automatically compensated by the difference of the water level between two electrode vessels. And T is the outlet tube for gasses formed by the electrode reaction only in the case of extremely long run. In our experiments, however, we found little gasses on an electrode during run.

Supporting material. As a supporting material we used the cellulose powder described by Flodin and Kupke[6]. We prepared the powder by refluxing good quality clean cotton in absolute methanol containing 1 M hydrogene chloride for about twenty hours. The properties of this powder are as excellent as not to disturb electrophoresis, elution and protein determination. In contacting with water for a long time, however, the powder has a tendency to be destroyed into a fine powder which disturbs protein determination. Therefore, for stocking the powder, it should be kept in dry state, because of its fragility. It is always better to use the new powder than that used in a zone electrophoresis for a long time.

Packing of the column. The procedure, by which the cellulose powder is to be well packed in A, is similar to that described by Flodin and Kupke[6]. An adaptor is attached to the lower end of A. A long glass tube (2~3 m) with the same diameter as that of A is jointed to the upper end of A, and a 1.5 l funnel is connected to the opposite end of a long tube by a rubber tubing. Before packing the column, it is necessary to keep the column and glass tube vertical, to fill the tubes with the conducting buffer solution and to avoid air bubbles in the glass filter. When a suspension of the cellulose powder in the buffer solution (1 : 10 wet volume) is poured into the funnel, stopcock of the adaptor is opened so that the rate of outflow of the solution can be controlled to 3 drops per second as the column builds up. Then powder is settled evenly to form a flat rising boundary of the packed material. When the buffer level comes down as far as the sharp upper surface of the packed powder, the flow of liquid through the column is stopped by closing the stopcock of the adaptor. After these procedures the long tube is carefully removed from A. At this time precaution should be taken to keep the surface of the packed powder below that of the buffer solution.

Application of the initial zone. When applying the sample solution to be analyzed, care must be taken that the initial zone is not spoiled during application. Hereupon, a glass filter fitting the inside of A is gently placed on the packed surface, and then the buffer solution on the glass filter is allowed to drain very slowly through A. Just when the buffer surface coincides with that of the glass filter the sample is applied on the glass filter by dropwise addition. By the above treatment no disturbance of the packed cellulose surface is found. All the sample solution having been applied, the flow is started again by opening the stopcock of the adaptor and the flow rate is controlled to 10 ml per hour. At the moment the sample solution enters downward from the surface, the flow is stopped and a few ml of the buffer solution is added in the same way as application of the sample solution.

Zone electrophoresis. After A is connected to D, whose space is filled with the buffer, the oppening (M) is turned to close and the stopcock (L) is shut, the adaptor at the lower end of A is detached without allowing the buffer solution to flow through the column. Then all the parts are jointed together as shown in Fig. 1. Whole apparatus is filled with the buffer solution. After M is turned to open position, zone electrophoresis is started.

Elution. At the end of zone electrophoresis, M is closed and A together with D is removed from the whole apparatus. Then the adaptor is attached to the lower end of A again. The eluate is collected by a fraction collector. During elution, the column is cooled by cold water passing through F. The elution rate is controlled to 12 ml per hour at 5°C. Each test tube contains 2.5 ml of the eluate.

RESULTS AND DISCUSSION

Boundary electrophoresis. Prior to moving

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[6] P. Flodin and D.W. Kupke, *Biochim. et Biophys. Acta*, **21**, 368 (1956).

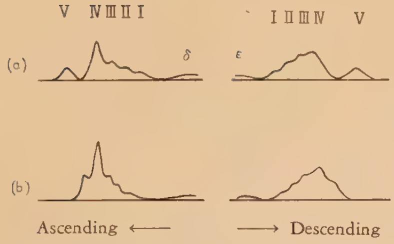


FIG. 4. Boundary Electrophoresis of Crystalline Yeast PGA Mutase in Veronal and Phosphate Buffers.

In the case of (a) boundary electrophoresis was carried out in a veronal buffer of pH 7.0 and 0.1 ionic strength at a potential gradient of 4.05 volts per cm for 18,000 seconds. In the case of (b), in a phosphate buffer of pH 7.0 and 0.1 ionic strength at a potential gradient of 4.6 volts per cm for 10,860 seconds. The patterns were obtained from a 2.8 per cent protein solution at 0.7°C, respectively. The numbers refered to the component.

boundary electrophoresis, three times recrystallized yeast PGA mutase was dissolved in a veronal buffer of pH 7.0 and 0.1 ionic strength to a concentration of approximately 4 per cent protein solution, and dialyzed against the same buffer for twenty four hours in an ice box. Boundary electrophoretic analysis was performed in the same buffer at a potential gradient of 4.05 volts per cm for 18,000 seconds (0.7°C). The pattern is shown in Fig. 4.

From these results, it was found that the crystalline enzyme was composed of five components. In a phosphate buffer (pH 7.0, $\Gamma/2=0.1$), the similar results were obtained, but the mobility of each component was greater than that in the case of a veronal buffer. Though we reported that PGA mutase was composed of 6 components in previous paper[2)], we found 5 components in this experiment. It may be due to differences among batches of yeast used as the starting materials or incubation times.

Vertical zone electrophoresis. The crystalline enzyme was dissolved in a veronal buffer of pH 7.0 and 0.068 ionic strength, and dialyzed against the same buffer before vertical zone electrophoresis was performed. Four ml of the dialyzed enzyme solution containing 250 mg of protein was applied on the electrophoresis column. A potential difference of 250 volts between the two electrodes was then applied for 150 hours at 6°C (40 mA). When run was finished, elution was carried out. Every effluent was collected into test tubes by a fraction collector. These results are shown in Fig. 5.

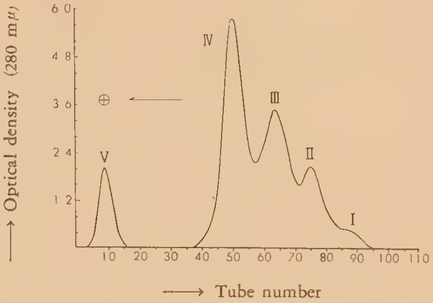


FIG. 5. Electrophoregram of Crystalline Yeast PGA Mutase by Means of Vertical Zone Electrophoresis.

Run (6°C) was performed in a veronal buffer of pH 7.0 and 0.068 ionic strengtn for 150 hours at 40 mA. 250 mg of the yeast crystalline enzyme was applied. Each tube contained 2.5ml. The numbers refered to the component.

This pattern is analogous to the ascending one in boundary electrophoretic analysis. Hereupon, the complete separation of each component from the crystalline preparation in a large quantity has been established. The specific activity of each component is given in Table I.

Among the five components, four of them (components I to IV) are the PGA mutase protein, whereas component V has no activity. The specific activity of component I is the highest and the others become lower in order.

Without the addition of the coenzyme, each component revealed one-ninth activity as much as that in the presence of the coenzyme. Therefore, a small amount of the coenzyme seemes to be bound to PGA mutase protein as described before[2)].

Crystallization of each component separated. We succeeded in the crystallization of each component by the dropwise addition of saturated ammonium sulfate solution to the solution containing each component at 3°C in the same way as preparing the original crystals. Prior to the crystallization we chose test tubes in which only one component was contained. These crystals are shown in Fig. 6.

TABLE I. ACTIVITY WITHOUT ADDITION OF COENZYME AND SPECIFIC ACTIVITY OF EACH COMPONENT.

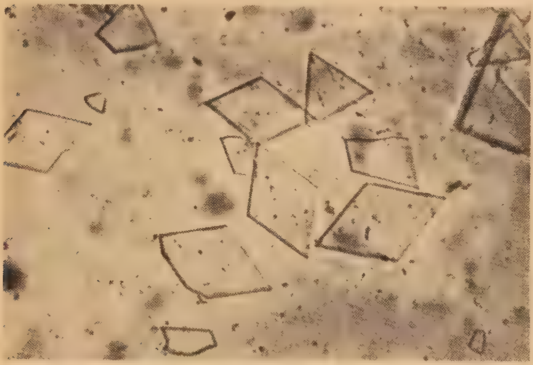
Component	Activity* without Addition of Coenzyme	Specific Activity**
Original	220	1,800
I	523	4,060
II	410	3,030
III	272	2,240
IV	35	200
V	0	0

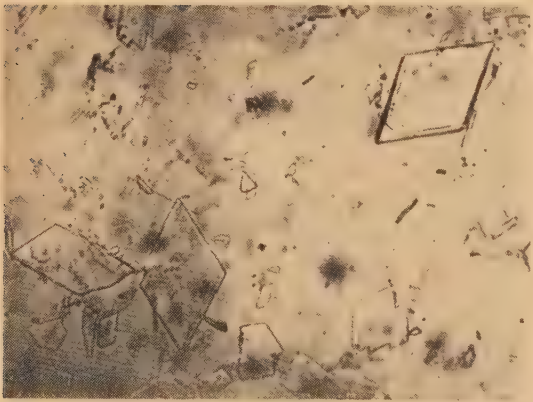
* Micromoles of D-3PGA formed per ten minutes per mg of the enzyme protein. The conditions of reaction were the same as those for the standard assay except that the coenzyme was omitted.

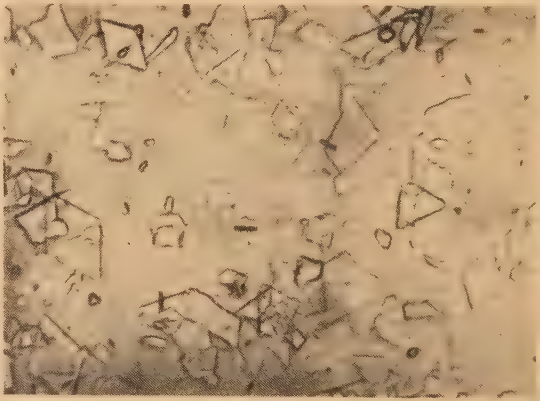
** Units per mg of the enzyme protein under the conditions of the standard assay8).

From these results, it is obvious that the crystalline form and the axial ratio of each crystal are the same. Moreover, every crystal is the same as the original one. These facts suggest that there is no essential difference among the proteins of these components. In order to interpret the difference among the mobilities of individual components, it is necessary to understand their molecular weights, forms and net charge distributions. As the original crystalline preparation sediments as a single peak in ultracentrifugal analysis1), molecular weights of individual components are assumed to be similar. Therefore the different mobilities may be caused by different net charges. Hereupon, it appears reasonable to assume that these different mobilities are due to the difference among amide contents of the several components.

Now we are intending to clarify the reason why the specific activity and the mobility of each component differs from the others.


Component I


Component II


Component III

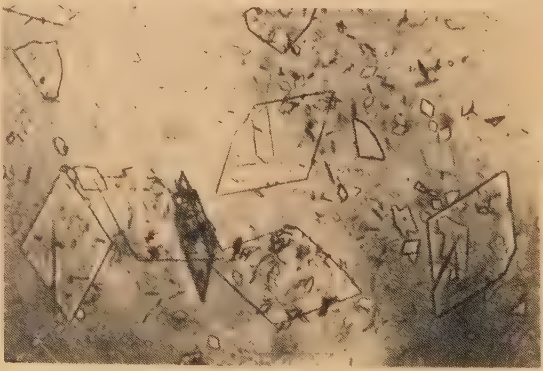

Component IV

FIG. 6. Crystal of Each Component

SUMMARY

Boundary electrophoretic behaviors of crystalline yeast PGA mutase have been studied in veronal and phosphate buffers. The crystalline enzyme used here is composed of five components.

Each component has been separated in a large quantity by using the special vertical zone electrophoresis apparatus with Hg-Hg_2Cl_2 reversible electrodes, which is deviced to compensate electroosmotic flow automatically.

The components I to IV are the PGA mutase protein and have different specific activities.

Each separated component has been crystallized by the dropwise addition of saturated ammonium sulfate solution.

The crystalline form of each component separated is the same as the original one.

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(*continued from front cover*)

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